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Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: Characterization and comparison with the actions of interleukin 1

(inflammation/coagulation/tissue factor/monokine/endotoxin)

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ABSTRACT Human recombinant tumor necrosis factor (rTNF) was found to act directly on cultured human vascular endothelium to induce a tissue factor-like procoagulant activity (PCA). After a 4-hr incubation in rTNF (100 units/ml), serially passaged endothelial cells isolated from umbilical veins, saphenous veins, iliac arteries, and thoracic aortae demonstrated a dramatic increase (4- to 15-fold, 21 experiments) in total cellular PCA as measured with a one-stage clotting assay. rTNF-induced PCA was also expressed at the surface of intact viable endothelial monolayers. Induction of PCA by rTNF was concentration dependent (maximum, 500 units/ml), time dependent, reversible, and blocked by cycloheximide and actinomycin D, and it occurred without detectable endothelial cell damage. Actions of rTNF were compared with those of natural human interleukin 1 (IL-1) derived from stimulated monocytes and two distinct species of recombinant IL-1, each of which also induced endothelial PCA. The use of recombinant polypeptides and specific neutralizing antisera established the distinct natures of the mediators. The kinetics of the endothelial PCA responses to TNF and IL-1 were similar, demonstrating a rapid rise to peak activity at ≈4 hr, and a decline toward basal levels by 24 hr. This characteristic decline in PCA after prolonged incubation with TNF or IL-1 was accompanied by selective endothelial hyporesponsiveness to the initially stimulating monokine. Interestingly, the effects of TNF and IL-1 were found to be additive even at apparent maximal doses of the individual monokines. Endothelial-directed actions of TNF, alone or in combination with other monokines, may be important in the initiation of coagulation and inflammatory responses *in vivo*.

In 1975 Carswell *et al.* (1) described an activity in the serum of mice primed with bacillus Calmette–Guerin and treated with endotoxin that caused "hemorrhagic necrosis" of certain tumors. This activity was termed "tumor necrosis factor" (TNF) and has become the subject of extensive research (2), leading to its identification as a monokine, purification and biochemical characterization (2–7), and the cloning and expression of its gene (8–11). Although initially it was thought that TNF acted only on tumor cells, it has become clear that certain nontumor cells possess TNF receptors (12–14) and may be targets for other biological activities of this monokine. In separate investigations, Cerami and coworkers described a product of endotoxin-stimulated murine macrophages, termed "cachectin," which suppresses the synthesis of lipoprotein lipase by adipocytes (15–17). Recently, it has been established that cachectin and TNF appear to be identical (18, 19) and that a specific antiserum to cachectin/TNF can protect mice from the lethal effects of endotoxin (20).

We now report that human recombinant TNF (rTNF) is a potent inducer of procoagulant activity (PCA) in human endothelial cells cultured from various blood vessels. We compare the effects of rTNF with those of human monocyte-derived interleukin 1 (hmIL-1) and two distinct species of recombinant human interleukin 1 (rIL-1). We have previously shown that hmIL-1 acts on human endothelial cells to induce a tissue factor-like PCA (21–23), to dramatically increase the adhesiveness of their surfaces for leukocytes (23, 24), and to stimulate the expression of a new endothelial surface antigen (25). Our present data suggest that TNF, alone or in combination with interleukin 1 (IL-1), can significantly influence hemostatic/thrombotic activities at the blood-vascular wall interface. Further, endothelial-directed actions of TNF may be relevant to intravascular coagulation and hemorrhagic tumor necrosis.

MATERIALS AND METHODS

Cell Cultures. Human umbilical vein endothelial cells (HUVEC) were isolated from two to five cord segments, pooled, and grown in primary culture in medium 199 (M199, M. A. Bioproducts, Walkersville, MD) with 20% fetal calf serum (GIBCO, Grand Island, NY, or HyClone, Logan, UT) and antibiotics, as previously described (26). Cultures of HUVEC and other human endothelial cells isolated from adult saphenous veins (kindly provided by P. Libby, Tufts University Medical School, Boston) and adult iliac arteries and thoracic aortae (kindly provided by R. Weinstein, St. Elizabeth Hospital, Boston) were serially passaged (1:3 split ratios) in M199/20% fetal calf serum (HUVEC) or M199/10% fetal calf serum (others) supplemented with endothelial cell growth factor (50–150 μg/ml; a gift of T. Maciag, Meloy Laboratories, Springfield, VA) and porcine intestinal heparin (50–100 μg/ml, Sigma) (27) in Costar (Cambridge, MA) tissue culture flasks (75 cm^2) coated with purified fibronectin (1–10 μg/cm^2, Meloy Laboratories, Springfield, VA) or 0.1% gelatin (Bactogelatin 0143-02, Difco). Most experiments were performed with HUVEC at passages 2–4. A line of simian virus 40-transformed human endothelial cells, which had been established in this laboratory (28), was maintained in M199/15% fetal calf serum. For experimental use, cells were typically plated (2–4 × 10^4 cells per well) and grown to confluence (3–7 days) on the bottom of 16-mm diameter

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Abbreviations: HUVEC, human umbilical vein endothelial cell(s); PCA, procoagulant activity; TNF, tumor necrosis factor; rTNF, recombinant TNF; IL-1, interleukin 1; hmIL-1, human monocyte-derived IL-1; rIL-1, recombinant IL-1; U, unit(s).

tissue culture wells (Cluster 24, Costar) that had been coated with fibronectin (1–10 $\mu g/cm^2$).

Cytokines and Antisera. Human rTNF was expressed from a cDNA clone in *Escherichia coli* and purified to homogeneity (11) [1.3 mg of protein per ml in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline; 1.9×10^7 units (U)/mg of protein in L929 cytotoxicity assay (11)]. Natural hmIL-1, isolated as a mixture of two (or more) polypeptides (≈17 kDa) from the supernatant of *Staphylococcus albus*-stimulated monocytes by immunoabsorption and Sephadex chromatography (29), was obtained from Genzyme (Boston). This material was provided in sterile 0.15 M NaCl with 5% fetal calf serum and was reported to contain thymocyte costimulation activity (32) at 100 U/ml, <1.0% T-cell growth factor, interferon at ≤1 U/ml, and undetectable endotoxin activity. Two distinct species of human rIL-1, expressed from different complementary DNAs (cDNAs) in *E. coli* and termed rIL-1α and rIL-1β (30), were purified (10^8 U/mg of protein in thymocyte costimulation assays) and supplied as 1000 U/ml in sterile phosphate-buffered saline containing 0.1% bovine serum albumin (Genzyme). Each of the IL-1 preparations was active, and rTNF was inactive, in a comitogenesis assay using the D10.G4.1 T cell line (31) (courtesy of A. Abbas, Brigham and Women's Hospital, Boston). A rabbit antiserum was prepared to purified rTNF (anti-rTNF), and the immunoglobulin fraction of a rabbit antiserum to hmIL-1 (anti-hmIL-1) was purchased from Genzyme. These antisera neutralized the appropriate monokine in standard bioassays (TNF, L929 cytotoxicity; IL-1, thymocyte costimulation).

Cell Treatments. Cell monolayers were washed three times, incubated for up to 60 min at 37°C, and washed again prior to treatment with TNF or IL-1 in 0.5 ml of pretreatment media. Several pretreatment media, including RPMI 1640 (M. A. Bioproducts) with 10% fetal calf serum (RPMI/10% FCS) or 0.1% bovine serum albumin (RPMI/0.1% BSA), were tested and produced comparable results. After incubation (37°C, 5% CO_2) for up to 28 hr, each well was washed three times in 0.5 ml of RPMI 1640 medium and prepared for evaluation of PCA.

In certain experiments, cycloheximide (10 $\mu g/ml$) or actinomycin D (5 $\mu g/ml$) (Sigma) was added to the cultures 30 min prior to the addition of cytokines and allowed to remain throughout the pretreatment phase. In pilot experiments, this concentration of cycloheximide blocked greater than 97% of [^{35}S]methionine incorporation into endothelial monolayers.

Evaluation of Endothelial PCA. To determine total cellular PCA, a standard one-stage clotting (plasma recalcification) assay was performed as described (21) at 37°C, using glass tubes containing 100 μl of citrate-treated, pooled, normal donor, platelet-poor plasma, or coagulation factor VII-, IX-, or X-deficient plasma (George King Bio-Medical, Overland Park, KS) to which 100 μl of cell lysate (frozen-thawed three times, scrape harvested) and 100 μl of $CaCl_2$ (30 mM) were added. In certain experiments, cell surface-expressed PCA was assayed directly in the culture wells on intact viable monolayers, using a modified clotting assay (21). Milliunits (mU) of PCA were defined by standard curves developed with rabbit brain thromboplastin (Sigma) and normal, platelet-poor, citrate-treated human plasma; 10^3 mU of PCA corresponded to a clotting time of 20 sec in the standard assay.

RESULTS

rTNF Induces Procoagulant Activity in Cultured Human Vascular Endothelium. Human endothelial cells, which were isolated from neonatal umbilical cord veins, adult saphenous veins, adult iliac arteries, or adult thoracic aortae and maintained under standard culture conditions, contained low levels of PCA (Table 1). After a 4-hr incubation with human rTNF at 100 U/ml each of these endothelial cell types exhibited a dramatic increase in total cellular PCA (4- to 15-fold, 21 experiments) (Table 1). In contrast, a strain of simian virus 40-transformed umbilical vein endothelial cells (28), which constitutively expresses high levels of PCA (greater than 500 U/10^5 cells), was not significantly influenced by treatment with rTNF (5 ± 5% above control, mean ± SD, 3 experiments).

The effect of rTNF on endothelial PCA was found to be concentration dependent, time dependent, and reversible. Incubation of human endothelial monolayers for 4 hr with rTNF concentrations as little as 0.8 U/ml consistently increased PCA over basal levels (1.8- to 4.5-fold, $P < 0.005$, 6 experiments), and rTNF at 500 U/ml resulted in near maximal stimulation (8.1- to 18.8-fold, 6 experiments) (Fig. 1). As seen in Fig. 2, the kinetics of the endothelial-PCA responses to TNF and IL-1 were similar. Continuous exposure of HUVEC to either rTNF (500 U/ml) or hmIL-1 (5 U/ml) led to an increase in total cellular PCA, which was first detected at 30–60 min, peaked at ≈4 hr, and declined toward

Table 1. rTNF induces PCA in cultured human endothelial cells isolated from various vessels

Endothelial cell source (vessel)	n	PCA, mU/10^5 cells	
		Control	rTNF
Umbilical vein	11	36 ± 4	308 ± 38
Saphenous vein	6	40 ± 10	307 ± 65
Iliac artery	3	46 ± 20	238 ± 67
Thoracic aorta	1	49 ± 9	247 ± 21

Serially passaged (passages 2–8) human vascular endothelial cells isolated from neonatal umbilical veins and adult saphenous veins, iliac arteries, or thoracic aortae were grown to confluence in 16-mm diameter tissue culture wells. In each of n separate experiments, replicate cultures were incubated for 4 hr in pretreatment media (usually RPMI/10% FCS) without (control) or with rTNF at 100 U/ml and assayed for total cellular PCA. Data represent mean ± SEM.

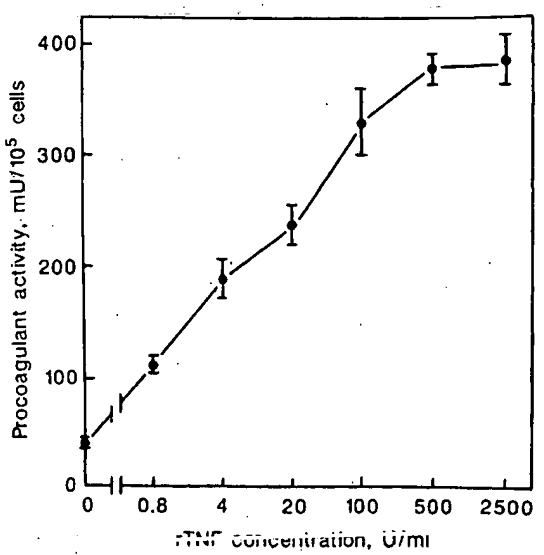

FIG. 1. Concentration dependence of rTNF induction of total cellular PCA in human endothelial cells. PCA was assayed in freeze–thaw lysates of HUVEC (passage 2) after a 4-hr incubation with rTNF at the indicated concentrations. Data represent mean ± SD from three cultures. Similar concentration dependence was observed in four additional experiments on HUVEC and in a single experiment on human endothelial cells isolated from saphenous veins and thoracic aortae.

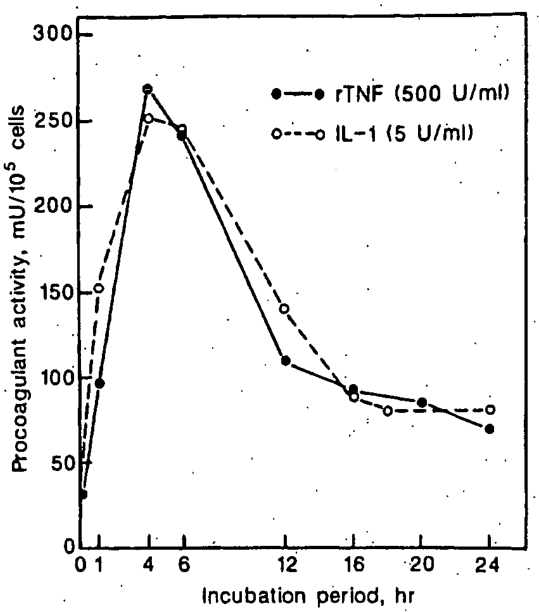

FIG. 2. Time course of rTNF and hmIL-1 induction of PCA in HUVEC (passage 2). Replicate cultures were incubated in RPMI/10% FCS with rTNF or hmIL-1 for defined periods up to 24 hr. Each point represents the mean of triplicate cultures. Variation within each experimental group was less than 10%. Similar results were obtained in two additional experiments.

basal level by 24 hr. Brief exposure (15 min) of the cultures to either rTNF at 100 U/ml or hmIL-1 at 5 U/ml, followed by washing, resulted in substantial increases in PCA by 6 hr (not shown). PCA in endothelial cultures that had been treated with rTNF at 100 U/ml for 4 hr, washed, and incubated in the absence of rTNF for an additional 22–24 hr returned to near basal levels. Rechallenge of these cultures with fresh rTNF (100 U/ml) or hmIL-1 (5 U/ml) resulted in a second rise in PCA, which was equivalent (100 ± 14% and 111 ± 10%, mean ± SD, 3 experiments) to that obtained by primary stimulation of nonpretreated monolayers. Induction of endothelial PCA by rTNF or hmIL-1 was blocked by cycloheximide at 10 μg/ml (81 ± 9% and 85 ± 13% inhibition, mean ± SD, 5 experiments) or actinomycin D at 5 μg/ml (107 ± 13 and 102 ± 2% inhibition, 5 experiments), indicating the need for *de novo* protein and RNA synthesis.

Two Distinct Species of rIL-1 Induce Endothelial PCA. Recently, it has been established that hmIL-1 consists of several biochemically distinct species (29, 32), which appear to be encoded by at least two different genes (30, 33). We recently reported that a lysate of L cells that expressed the cDNA of a species of human IL-1 cloned by Auron *et al.* (33) (a gift of T. Livelli, Cistron, Pine Brook, NJ) also induced PCA in cultured endothelium (23). In addition, purified rIL-1 (a gift of C. Dinarello, Tufts University Medical Center, Boston) produced by expression of this same cDNA in *E. coli* was also active in our assay (unpublished observations). In the current studies, we have examined the effects of two *E. coli*-derived purified rIL-1 polypeptides termed rIL-1α and rIL-1β (30). rIL-1α is homologous to a previously described murine IL-1 species (34) and is structurally distinct from rIL-1β. The cDNA sequence of rIL-1β (30) is identical to that previously reported by Auron *et al.* (33) and appears to correspond to the predominant species (pI = 7) produced by stimulated human monocytes (29, 30). As seen in Table 2, both rIL-1α and rIL-1β induced endothelial PCA. The effects of these distinct species of rIL-1 were also found to be concentration dependent, time dependent, and reversible

Table 2. Specific antisera block the induction of endothelial PCA by rTNF, hmIL-1, rIL-1α, and rIL-1β

Endothelial cell treatment	PCA, mU/10⁵ cells: No antibody addition	Anti-rTNF	Anti-hmIL-1
Control	14 ± 1	15 ± 1	17 ± 1
rTNF	182 ± 13	13 ± 1	140 ± 5
hmIL-1	191 ± 8	190 ± 8	20 ± 1
rIL-1α	221 ± 9	241 ± 18	70 ± 10
rIL-1β	201 ± 5	202 ± 12	24 ± 1

Rabbit antiserum to rTNF (anti-rTNF) or the immunoglobulin fraction of a rabbit antiserum to hmIL-1 (anti-hmIL-1) was incubated (1:600 and 1:15 final dilution, respectively) with control media (RPMI/10% FCS) or the indicated mediator (rTNF, 100 U/ml; hmIL-1, 2.5 U/ml; rIL-1α and rIL-1β, activities equal to 2.5 U/ml of hmIL-1 in D10.G4.1 comitogenesis assay) in RPMI/10% FCS for 2 hr at 37°C and transferred to replicate HUVEC cultures (passage 3). After 4 hr of incubation, the cultures were assessed for total cellular PCA. Normal rabbit serum and the immunoglobulin fraction of normal rabbit serum did not significantly inhibit monokine induction of PCA (not shown). Data represent mean ± SD from triplicate wells. Similar results were found in three additional experiments.

(not shown). TNF and IL-1 preparations could be inactivated by heat (100°C, 15 min; 92–102% inhibition, 4 experiments) and were unaffected by the presence of polymyxin B (50 μg/ml; 0–11% inhibition, 3 experiments). These observations distinguish the action of the monokines from that of endotoxin, which also can induce endothelial PCA (23, 35–37).

A rabbit antiserum prepared to rTNF abolished the PCA-inducing activity of rTNF but did not inhibit the actions of hmIL-1, rIL-1α, or rIL-1β (Table 2). Conversely, a second antiserum prepared to hmIL-1 blocked the induction of PCA by all the preparations of IL-1 tested without significantly inhibiting rTNF activity. These observations help to establish that (*i*) the PCA-inducing activity of each preparation is attributable to a specific monokine rather than some other component, and (*ii*) TNF is not a significant contaminant of the purified natural hmIL-1 preparations used in our studies.

Effects of Combined and Sequential Treatments with rTNF and IL-1 on Endothelial PCA. The effects of combined TNF and IL-1 treatments of endothelial monolayers were found to be additive even at apparent maximal concentrations of the individual monokines. As seen in Fig. 3, incubation of HUVEC with increasing concentrations of rTNF in the presence of hmIL-1 at 5 U/ml resulted in a greater increase in PCA than did incubation with rTNF alone.

We previously demonstrated (21) that continuous incubation of endothelial monolayers in hmIL-1 at 5 U/ml for 24 hr led to a state of hyporesponsiveness to rechallenge (4 hr) with fresh hmIL-1. In the current experiments, after incubation for 24 hr in rTNF (100 U/ml), the addition of fresh rTNF (100 U/ml) resulted in only a 60 ± 20% increase in PCA (mean ± SEM, 4 experiments). However, the addition of hmIL-1 at 5 U/ml to replicate monolayers that had been treated for 24 hr with rTNF (100 U/ml) resulted in a 420 ± 70% stimulation (4 experiments). Similarly, endothelial cultures that were pretreated with IL-1 for 24 hr and then rechallenged with TNF demonstrated a significantly greater response than those cultures that were rechallenged with IL-1 (not shown).

Characterization of TNF- and IL-1-Induced Total Cellular and Cell Surface PCA. The nature of the PCA induced in endothelial cells by rTNF and IL-1 was investigated by using human plasmas deficient in specific coagulation factors. In our standard one-stage clotting assay, total cellular endothelial PCA that had been induced by rTNF or hmIL-1, alone or in combination, was expressed in normal human plasma and factor IX-deficient plasma but not in plasmas that were

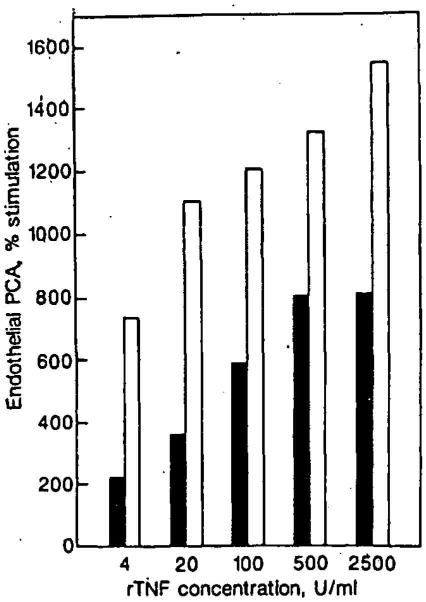

FIG. 3. Induction of PCA in human endothelial cells by combined rTNF and hmIL-1 treatment. PCA was assayed in freeze-thaw lysates of HUVEC cultures (passage 3) after a 4-hr incubation with rTNF alone (solid bars) at the concentrations shown or with rTNF in combination with hmIL-1 at 5 U/ml (open bars). Percent stimulation was calculated from triplicate wells for each experimental group as [(monokine-stimulated PCA − control PCA)/control PCA] × 100. hmIL-1 (5 U/ml) alone promoted a 770% stimulation of endothelial PCA. Similar results were obtained in three additional experiments with hmIL-1 and in two experiments with rIL-1α and rIL-1β.

deficient in factor VII or factor X (Table 3). In a modified clotting assay, unstimulated intact monolayers of HUVEC displayed essentially no surface-available PCA (clotting times ≈500 sec). However, incubation of these cells for 4 hr in rTNF (200–500 U/ml) alone or in combination with hmIL-1 at 5 U/ml resulted in a marked increase in surface PCA (clotting times 110–140 and 55–85 sec, respectively, 2 experiments). Cell surface PCA that had been induced by rTNF, hmIL-1, or both was not expressed in coagulation factor-VII or factor X-deficient plasma but was expressed in factor IX-deficient plasma (not shown). Incubation of endothelial monolayers with rTNF (0.8–500 U/ml) or hmIL-1 (0.5–10 U/ml) for up to 24 hr did not result in apparent cell damage as assessed by trypan blue exclusion (97–101% of controls, 3 experiments). These studies suggest that monokine-induced total cellular and cell surface endothelial PCA primarily involves a tissue factor-like activity. However, actions of TNF and IL-1 on other procoagulant and/or anticoagulant functions of vascular endothelium are not excluded in the current studies.

Table 3. Characterization of TNF- and IL-1-induced endothelial PCA

Endothelial treatment	PCA, mU/10^5 cells			
	Normal plasma	Factor-deficient plasma		
		VII	X	IX
Control	16 ± 1	9 ± 1	<1	<1
rTNF	228 ± 9	8 ± 0	<1	134 ± 5
hmIL-1	217 ± 16	11 ± 1	<1	143 ± 5
rTNF + hmIL-1	486 ± 44	7 ± 0	<1	373 ± 42

Replicate cultures of HUVEC in 100-mm dishes were treated for 4 hr with RPMI/10% FCS alone (control) or supplemented with rTNF (500 U/ml), hmIL-1 (5 U/ml), or both, washed, and lysed (freeze–thaw). Standard one-stage clotting assays were performed on cell lysates using normal human plasma or specific coagulation factor-deficient human plasmas. Data represent mean ± SD of triplicate determinations. Similar results were obtained in two other experiments.

DISCUSSION

The vascular endothelium is capable of actively participating in coagulation and inflammatory events in a variety of ways (reviewed in ref. 38). In this report we have demonstrated that human rTNF acts on cultured human vascular endothelium to induce a tissue factor-like PCA. This effect of TNF, like that previously reported for IL-1 (21–23), was found to be concentration dependent, time dependent, reversible, and blocked by inhibitors of protein and RNA synthesis. Independence of the endothelial-directed actions of TNF and IL-1 was established by the use of recombinant species of these monokines and by inhibition with specific antisera.

TNF and IL-1 are both produced by mononuclear phagocytes after exposure to appropriate stimuli, including bacterial endotoxin. In our studies, modest concentrations of TNF and IL-1, in combination, were able to significantly increase endothelial PCA. Moreover, the effects of TNF and IL-1 on endothelial PCA were additive even at maximal doses of the individual monokines. These results, and our observations on the induction of selective endothelial hyporesponsiveness after a 24-hr incubation with the individual monokines, suggest that TNF and IL-1 actions on endothelial PCA may involve different mechanisms. Endotoxin itself can also stimulate endothelial PCA (23, 35–37). However, the effects of TNF and IL-1 in our experiments were distinguished from those of endotoxin by polymyxin and heat treatments and by specific antibody neutralization of the mediators. Interestingly, endothelial cells themselves have recently been shown to elaborate IL-1 like activities (39–41), which could play a role in the local regulation of vessel wall thrombogenicity *in vivo* (41).

In the current study, the endothelial PCA induced by TNF and IL-1, alone or in combination, primarily involved a tissue factor-like activity. However, it appears that these monokines can also influence other components of the "endothelial hemostatic/thrombotic balance" (38). For example, recent experiments indicate that IL-1 treatment of vascular endothelium also results in a decrease in its fibrinolytic activity (42), thus, potentially favoring the maintenance of fibrin at sites of inflammation. TNF and IL-1 also share at least two other endothelial-directed actions. First, like IL-1 (23, 24), TNF can increase the adhesiveness of endothelial cell surfaces for leukocytes (43, 44). Second, both monokines stimulate the expression of the same endothelial activation antigen (25), which can be found at sites of inflammation *in vivo* (45). However, not all endothelial responses to these monokines are identical. For example, long-term incubation (3–4 days) of endothelial cultures with TNF results in increased expression of class I major histocompatibility antigens (46), whereas IL-1 has no significant effect (unpublished observation).

Actions of TNF and IL-1 on vascular endothelium *in vivo* may contribute to the development of thrombotic and inflammatory reactions. In particular, we suggest that the effects of these monokines on endothelial PCA may be involved in the development of endotoxin-induced disseminated intravascular coagulation and hemorrhagic necrosis of tumors. To further understand the pathophysiology of TNF- and IL-1-mediated responses *in vivo*, it will be useful to examine human tissues for the expression of endothelial activation antigens (25). In addition, it may be important to investigate the actions of these monokines on various types of normal as

well as tumor-associated endothelium. Better understanding of the endothelial-directed actions of TNF and IL-1 may contribute to more effective therapeutic interventions in inflammatory and neoplastic diseases.

Note Added in Proof. Since submission of this article, Nawroth and Stern have reported (47) that rTNF treatment of cultured endothelial cells alters their hemostatic properties, in part, through the induction of tissue factor procoagulant activity.

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Procoagulant effect of the OKT3 monoclonal antibody: Involvement of tumor necrosis factor

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Procoagulant effect of the OKT3 monoclonal antibody: Involvement of tumor necrosis factor. We recently observed that the prophylactic administration of high doses of OKT3 monoclonal antibody (MoAb) in cadaveric renal transplantation favors the development of thromboses of the grafts' main vessels and of thrombotic microangiopathies. These clinical observations led us to perform sequential determinations of plasma levels of prothrombin fragment 1 and 2 (F 1 + 2) and fibrin degradation products (FDP) after the first injection of 5 or 10 mg OKT3 given as prophylaxis in kidney transplant recipients. The values observed have been compared with those of kidney transplant recipients not treated with OKT3. F 1 + 2 levels peaked four hours after the first injection of 5 mg OKT3 (mean ± SEM: 4.82 ± 0.73 vs. 1.75 ± 0.37 nmol/liter in controls, $P < 0.01$), indicating activation of the common pathway of the coagulation cascade. FDP levels were already above baseline values at four hours and continued to increase until 24 hours (mean ± SEM at 24 hr, 4729 ± 879 vs. 1038 ± 320 ng/ml in controls, $P < 0.05$), indicating a fibrinolytic process. The magnitude and the time course of the changes in F 1 + 2 and FDP plasma levels were similar whether the patients received 5 or 10 mg dose of OKT3. The levels of von Willebrand factor (VWF) antigen, a molecule released by activated or damaged endothelial cells, were also significantly increased after injection of OKT3 (mean ± SEM at 24 hr, 3.67 ± 0.18 vs. 2.17 ± 0.11 U/ml in controls, $P < 0.05$). The procoagulant effects of OKT3 were further investigated in vitro on human umbilical vein endothelial cells (HUVEC). It was found that OKT3 induces peripheral blood mononuclear cells (PBMC) to release soluble mediators that trigger the generation of thrombin at the HUVEC surface by a tissue factor-dependent mechanism. The addition of chimeric anti-TNF-α MoAb to culture supernatants of OKT3-stimulated PBMC strongly inhibited the thrombin generation in this model. We conclude that OKT3 activates the coagulation cascade in vivo and that TNF α is a mediator of the procoagulant effects of OKT3 at the endothelial cell level.

The first dose reactions observed after the administration of the OKT3 monoclonal antibody (MoAb) in transplant recipients include fever, chills, headaches and digestive symptoms [1]. Occasionally, more severe complications such as pulmonary edema and aseptic meningitis also occur. These adverse reactions appear to be related to the systemic release of cytokines, particularly of tumor necrosis factor-alpha (TNF-α) [2, 3]. We recently observed a new and severe complication of high-dose prophylactic OKT3 (10 mg/day) in kidney transplant recipients, namely the occurrence of intragraft thromboses [4]. In parallel, we found that the first injection of such a high dose of OKT3 is followed by a significant increase in the plasma levels of prothrombin fragment 1 + 2 (F 1 + 2), indicating activation of the common pathway of the coagulation system [4].

The present study was undertaken to further investigate the effects of OKT3 on hemostatic processes. First, we performed sequential determinations of plasma levels of F 1 + 2 and fibrin degradation products (FDP) after injection of a conventional (5 mg) or high (10 mg) dose of OKT3 in kidney transplant recipients. Second, we measured plasma levels of von Willebrand factor (VWF) antigen as a marker of endothelial cell activation. Third, we developed an in vitro model allowing to investigate the role of TNF-α in the procoagulant properties of OKT3 at the endothelial cell level.

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Methods

Patients

Adult recipients of cadaveric renal transplants were studied immediately before and during the first four or 24 hours post-transplantation. In the OKT3 groups, patients received an i.v. injection of 5 mg ($N = 10$) or 10 mg ($N = 7$) OKT3 (Orthoclone, Ortho Biotech, Raritan, New Jersey, USA) at the initiation of transplant surgery. Other immunosuppressive agents on the day of transplantation included azathioprine 2 mg/kg, and methylprednisolone 8 mg/kg given as an i.v. bolus three hours before surgery. In the control group, patients were immunosuppressed with azathioprine and methylprednisolone only during the first four hours ($N = 4$) or during the first 24 hours ($N = 3$) post-transplantation. As shown in Table 1, the immunological characteristics and the ischemia times were similar in the three groups of kidney transplant recipients.

In addition, we studied three patients who received a first injection of 10 mg OKT3 during the first trimester posttransplantation as anti-rejection therapy while they were under low dose prednisolone (0.3 to 0.5 mg/kg) and azathioprine (1 to 2 mg/kg), together with ($N = 1$) r without ($N = 2$) cyclosporin A (4 mg/kg). As in the prophylactic protocol, the OKT3 injection was preceded by an i.v. bolus of m thylprednisolone (8 mg/kg).

Table 1. Immun logical parameters and ischemia times

	Controls (N = 7)	OKT3 5 mg (N = 10)	OKT3 10 mg (N = 7)
No. of recipients with previous grafts	0/7	1/10	1/7
N . f recipients with anti-HLA antibodies	2/7	3/10	1/7
HLA incompatibilities			
A	0.57 ± 0.37	0.70 ± 0.21	0.57 ± 0.20
B	0.86 ± 0.34	0.30 ± 0.15	0.29 ± 0.18
DR	0.14 ± 0.14	0 ± 0	0.14 ± 0.14
Ischemia times (hours)	26.1 ± 2.2	22.5 ± 1.8	26.3 ± 3.2

Data are presented as mean ± SEM; differences between the groups are not statistically significant.

Blood collection

Blood samples were collected through a central venous catheter immediately before and 2, 4, 8 and 24 hours after the first injection of OKT3. In control patients, blood sampling was started at the initiation of surgery and followed the same timing as in OKT3 patients, except that four patients of this group could only be investigated during the first four hours because they subsequently received OKT3. After discarding the first 20 ml, blood was collected into vacuum tubes containing CTAD or tri-sodium citrate 3.8% as the anticoagulant (Diatube, Stago, Terumo Europe, Leuven, Belgium). Samples were kept on ice, centrifuged at 4000 rpm and plasma were stored at −70°C until assayed.

Hemostatic assays on plasma samples

Commercially available ELISA kits were used for determination of F 1 + 2 (Behringwerke, Marburg, Germany), FDP (Fibrinostika, Organon Teknika, Boxtel, Holland) [5] and VWF antigen (Stago, Franconville, France).

In vitro stimulation of peripheral blood mononuclear cells with OKT3

Peripheral blood mononuclear cells were isolated from blood btained from healthy volunteers by Ficoll-Hypaque density gradient centrifugation and cultured in 5% CO_2 atmosphere in M199 medium (Flow, Irvine, Scotland, UK) supplemented with 20% human serum, in the presence or absence of OKT3 10 ng/ml. Culture supernatants were collected after 24 hours and stored at −70°C until used in the assays described below.

Generation of thrombin at the surface of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) isolated according to the method of Jaffé [6] were cultured using M199 medium supplemented with 20% human serum, essential amino acids, endothelial cell growth factor (ECGF) (40 μg/ml), heparin (100 μg/ml), penicillin and streptomycin. Cells from the second passage (2 10^5 cells/ml) were transferred into 96 well plates and used at confluence after 24 hours in the absence of heparin and ECGF. After washing, 100 μl culture supernatants f PBMC were added, and the thrombin generated at the surface f HUVEC was determined by addition of calcium (100 μl, 30 mM, 2 min at 37°C) foll wed by normal human citrated plasma (45 sec at 37°C) before incubati n with the chromogenic substrate (S2238, Kabi Vitrum, 0.7 mM, 100 μl, 20 min at 37°C). The reaction was stopped with acetic acid (50 μl) and the absorbance red at 405 nm [7]. The amount of thrombin generated in this system was calculated using a standard curve obtained with purified thrombin and was expressed in mUnits of thrombin per 10^5 HUVEC. In some experiments, supernatants of OKT3-stimulated PBMC were incubated for two hours with different amounts of chimeric (human-mouse) anti-human TNF-α MoAb (provided by Centocor, Malvern, USA) or of an isotype-matched MoAb before to be incubated with HUVEC.

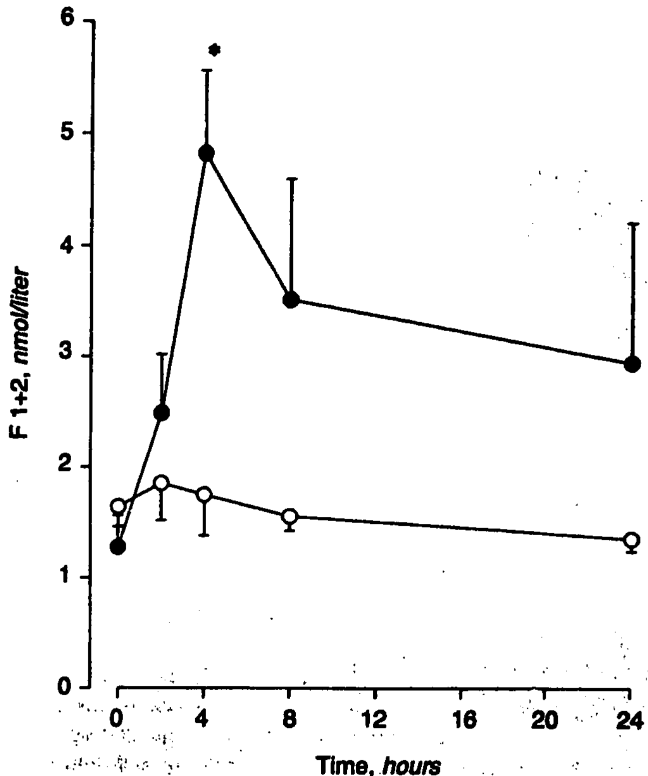

Fig. 1. *Evolution of F 1 + 2 plasma levels (mean ± SEM) after injection of 5 mg prophylactic OKT3 in kidney transplant recipients (N = 10) and in controls (N = 7 except for the 8 hr and 24 hr time points where N = 3).* Symbols are: (●) OKT 5 mg; (○) controls; $^{**}P \leq 0.01$.

Statistical analysis

Differences between groups were analyzed by Student's *t*-test.

Results

In vivo activation of the common pathway of coagulation by 5 mg OKT3

We measured F 1 + 2 and FDP plasma levels after the first injection of 5 mg OKT3 given prophylactically during transplantation surgery. As shown in Figure 1, a major increase in F 1 + 2 levels, reflecting activation of the common coagulati n pathway, was observed at four hours (mean ± SEM at 4 hr, 4.82 ± 0.73 vs. 1.75 ± 0.37 nmol/liter in controls, $P < 0.01$).

F 1 + 2 plasma levels returned to basal values within 24 hours except in two OKT3 patients. In one of them, thrombosis of the graft vein became apparent n the second postoperative day

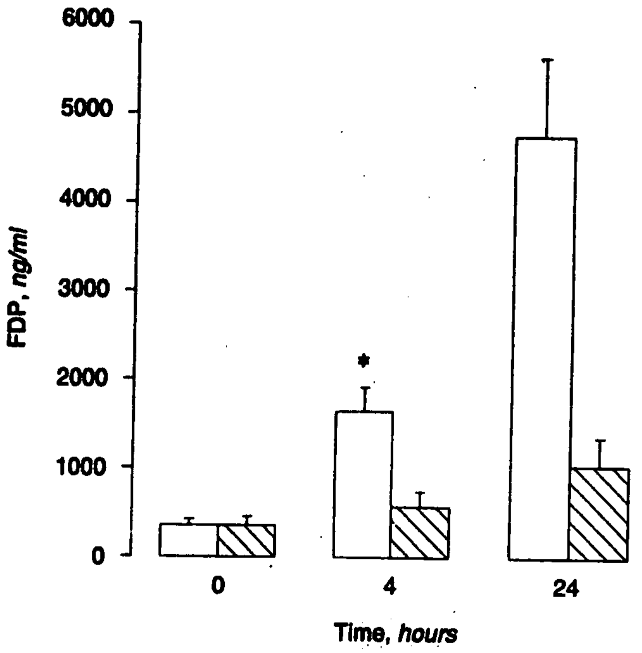

Fig. 2. *FDP plasma levels (mean ± SEM) before, 4 hours and 24 hours after injection of 5 mg prophylactic OKT3 in kidney transplant recipients (□, N = 10) and in controls (▧, N = 7 except at 24 hr where N = 3). *P < 0.05.*

Table 2. Peak plasma levels of F 1 + 2 and FDP levels according to OKT3 dosage

OKT3 dose	Peak plasma levels F 1 + 2 *nmol/liter*	FDP *ng/ml*
5 mg (N = 10)	4.82 ± 0.73	4729 ± 879
10 mg (N = 7)	5.88 ± 0.75	4422 ± 595

while the other developed a thrombotic microangiopathy diagnosed on the fifth post-operative day. The increase in F 1 + 2 levels was only observed after the first injection of OKT3. No significant changes were observed after the subsequent injections of the MoAb (data not shown).

As compared with controls, OKT3 patients also displayed increased FDP levels, indicating the occurrence of a fibrinolytic process (Fig. 2). This change was already apparent at four hours, but was more pronounced at 24 hours (mean ± SEM, 4729 ± 879 vs. 1038 ± 320 ng/ml in controls, $P < 0.05$).

As shown in Table 2, the changes in hemostatic parameters were essentially independent of the OKT3 dose. Peak plasma levels of F 1 + 2 were slightly higher in the patients who received 10 mg OKT3 but the difference did not reach statistical significance ($P = 0.411$).

Activation of coagulation was also evident when OKT3 was given as anti-rejection therapy. As shown in Figure 3, F 1 + 2 and FDP levels increased in the three patients studied with a kinetic similar to that observed under prophylactic OKT3.

Increased plasma levels of von Willebrand factor after injection of OKT3

As several cytokines released after the first injection of OKT3 might affect endothelial cells, we monitored plasma levels of VWF antigen [6]. Data represented in Figure 4 show that VWF plasma levels rose significantly following the injection of 5 mg prophylactic OKT3 (mean ± SEM at 24 hr, 3.67 ± 0.18 vs. 2.17 ± 0.11 U/ml in controls, $P < 0.05$).

OKT3 generates procoagulant activity at the surface of HUVEC

We next investigated the ability of OKT3 to induce thrombin generation at the surface of endothelial cells. For this purpose, HUVEC were incubated with OKT3 or with supernatants of PBMC stimulated by 10 ng/ml OKT3 before measurement of thrombin activity. We first observed that culturing HUVEC either with OKT3 alone (10 ng/ml) or with supernatants of unstimulated PBMC did not result in significant thrombin generation (Fig. 5). On the other hand, supernatants of OKT3-stimulated PBMC induce a massive generation of thrombin at the HUVEC surface. The magnitude and the time-course of this process was similar to that induced by recombinant TNF-α (10 ng/ml). The same experiments were repeated using factor VII-deficient plasma. Under this condition, supernatants of OKT3-stimulated PBMC did not induce any detectable thrombin generation, suggesting that this phenomenon was dependent on tissue factor expression (data not shown).

TNF-α is a mediator of the procoagulant effect of OKT3 at the endothelial cell level

Since supernatants of OKT3-stimulated PBMC contain high levels of TNF-α and because of the well-known procoagulant properties of this mediator, we investigated the role of TNF-α in the effects of OKT3 on HUVEC. As shown in Figure 6, the addition of a neutralizing chimeric anti-TNF-α MoAb to a culture supernatant of OKT3-stimulated PBMC (containing 1.9 ng/ml TNF-α) resulted in a dose-dependent inhibition of the thrombin generation. A 80% inhibition was achieved with 1 μg/ml chimeric anti-TNF-α MoAb whereas an isotype-matched control MoAb at the same concentration had no effect.

Discussion

The first observation of this study is that the first dose of 5 mg OKT3 in transplant recipients induces the activation of the common pathway of coagulation, as indicated by a significant rise in F 1 + 2 plasma level four hours after the injection of the MoAb. F 1 + 2 fragment is released during conversion of prothrombin to thrombin so that its measurement directly reflects activation of prothrombin [8]. In addition, the increase in FDP levels indicates that a fibrinolytic process also occurs after injection of OKT3. Although parameters of primary fibrinolysis were not measured, the late increase in FDP levels suggests that fibrinolysis after injection of OKT3 might, at least in part, be secondary to activation of the coagulation cascade.

Since our first observations of thrombotic complications consecutive to prophylactic OKT3 were made in patients receiving a high (10 mg) daily dose of the MoAb [4], we analyzed the impact of OKT3 dosage on hemostatic parameters. F 1 + 2 and FDP peak levels were similar whether the patients received the usual 5 mg dose or the high 10 mg dose of OKT3. This is in agreement with our most recent clinical data suggesting that, in kidney transplant recipients, 5 mg OKT3 treatment also carries

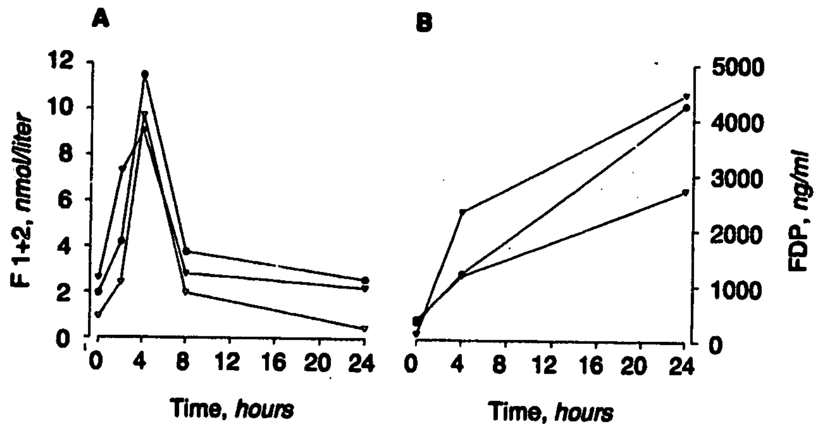

Fig. 3. *Evolution of F 1 + 2 plasma levels (A) and FDP levels (B) in three patients receiving a first injection of 10 mg OKT3 as antirejection therapy.*

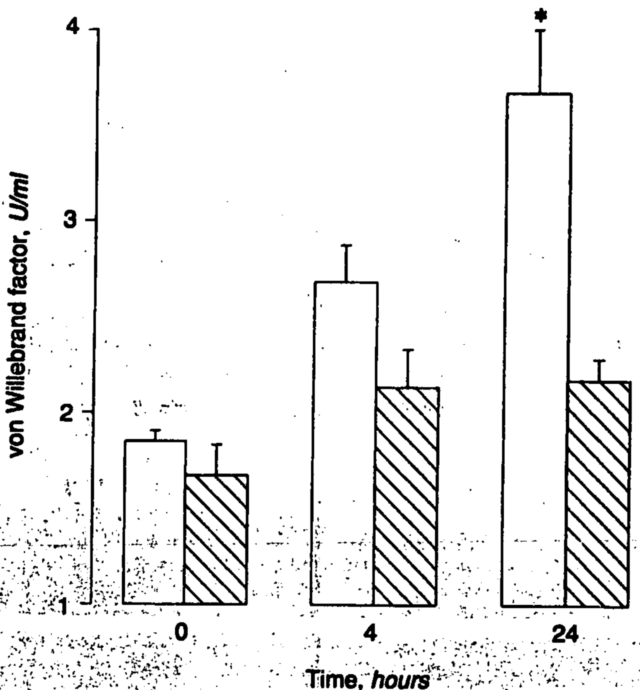

Fig. 4. *Plasma levels of VWF antigen (mean ± SEM) before, 4 hours and 24 hours after injection of 5 mg prophylactic OKT3 in kidney transplant recipients (□, N = 10) and in controls (▧, N = 7 except at 24 hr where N = 3). *P < 0.05.*

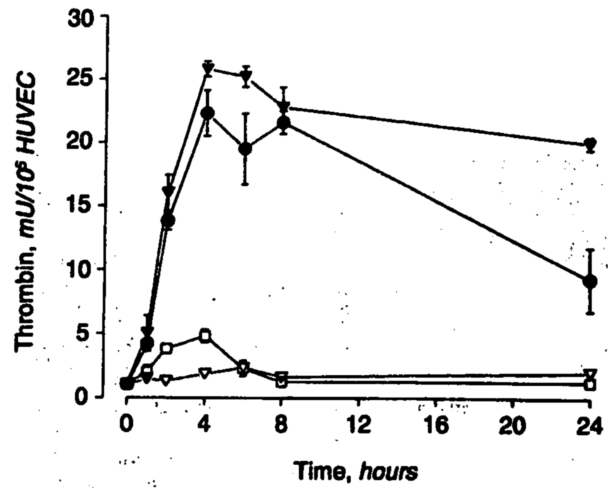

Fig. 5. *Thrombin generation (mean ± SEM of triplicate wells) at the surface of HUVEC at various times after incubation either with medium alone (▽), with supernatants of unstimulated PBMC (□), with supernatants of OKT3-stimulated PBMC (▼), or with recombinant TNF-α (10 ng/ml) (●).*

an increased risk of thrombotic complications, as illustrated in two patients of the present study.

It is likely that cytokines released in the circulation after the first injection of OKT3 play a central role in the procoagulant effect of the MoAb, as previously suggested by Kanfer et al [9]. Thus, interferon-γ has been shown to be involved in the induction of monocyte procoagulant activity by OKT3 in vitro [10]. Moreover, TNF-α is known to promote fibrin deposition and intravascular coagulation in vivo [11]. Interestingly, the magnitude and the time course of the changes in F 1 + 2 plasma levels in our patients were very similar to those reported by van der Poll et al in healthy volunteers injected with recombinant TNF-α [12]. In this setting, it is clear that the extrinsic route and not the contact system of coagulation was activated [12]. Endothelial cells could play an important role in this phenomenon since TNF-α stimulates the expression of tissue factor at their surface [13].

Our previous clinicopathological observations of thrombotic microangiopathy mimicking hemolytic-uremic syndrome as a complication of prophylactic OKT3 already suggested that endothelial cells are important targets of OKT3 toxicity [4]. The twofold increase in VWF levels 24 hours after injection of OKT3 is consistent with the hypothesis that endothelial cells are activated and/or damaged as a consequence of the administration of the MoAb. In vitro experiments on HUVEC allowed to determine more precisely the procoagulant effects of OKT3 at the endothelial cell level. While the MoAb has no direct effect on HUVEC, it induces PBMC to release soluble factors which trigger thrombin generation at the HUVEC surface by a tissue factor-dependent mechanism. The blocking effect of a chimeric anti-TNF-α MoAb revealed that TNF-α is a crucial mediator of the procoagulant effect of OKT3 in this

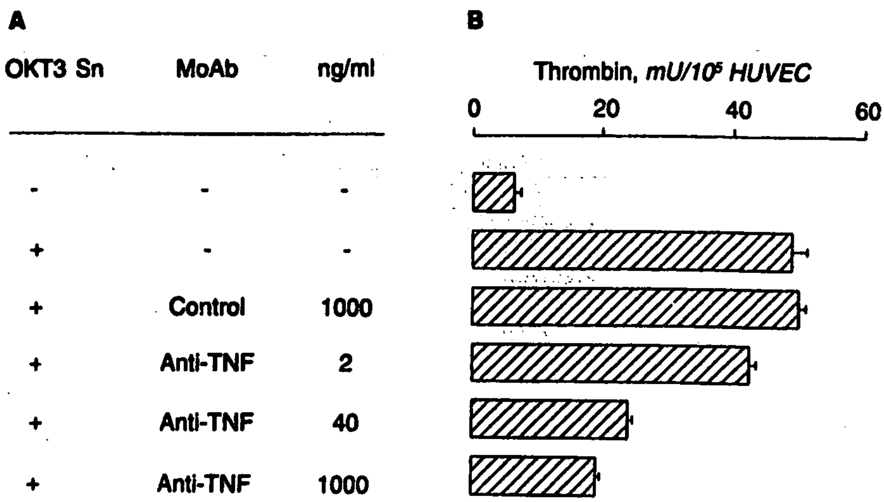

Fig. 6. *Role of TNF-α in the generation of thrombin at the HUVEC surface.* HUVEC were cultured with a supernatant of OKT3-stimulated PBMC in the presence of various concentrations of chimeric anti-TNF-α MoAb or of an isotype-matched control MoAb, and the generation of thrombin (mean ± SEM of triplicate wells) was measured after 4 hours.

system. This does not exclude the involvement of other cytokines, that is, interferon-γ, which are known to act in synergy with TNF-α in the induction of endothelial cell changes [11].

As far as the clinical relevance of our findings is concerned, one should first stress that the activation of coagulation is very transient and is only observed after the first injection of the MoAb. Interestingly, the release of cytokines also only occurs after the first injection OKT3 [2, 3]. In fact, the changes in hemostatic parameters parallel the release of cytokines which is maximal two hours after the first OKT3 injection. Since the main thrombotic complications in our experience occurred in patients receiving prophylactic OKT3 and involved intragraft vessels, it is possible that endothelial damages induced by ischemia and/or surgery contribute to the development of thromboses. Although thrombotic complications have not been reported as yet in patients receiving OKT3 as anti-rejection therapy, one should be aware that activation of coagulation also occurs in this setting.

Since TNF-α appears as an essential mediator of the procoagulant activity of OKT3, the irreversible thrombotic complications induced by prophylactic OKT3 could possibly be prevented by neutralization of TNF-α or abrogation of its release. This might represent the rationale for a trial of anti-TNF-α MoAb in patients receiving OKT3 as well as for the clinical development of non-activating OKT3-like MoAb [14].

Acknowledgments

This work was supported by the Fonds de la Recherche Scientifique Médicale (Belgium) and by Cilag Benelux. We thank Dr. Wendy Dixon (Centocore, Malvern, USA) for providing the chimeric anti-TNF-α MoAb. The technical assistance of Claude Habran and Alain Crusiaux is gratefully acknowledged.

Reprint requests to Michel Goldman, M.D., Hôpital Erasme, Department of Immunology, Hematology, Transfusion, 808 route de Lennik, B-1070 Brussels, Belgium.

Note added in proof

Since the submission f this manuscript, another study has demonstrated activation of coagulation and fibrinolysis following OKT3 administration [RAASVELD MH, HACK CE, TEN BERGE IJ: Thromb Haemostas (in press)].

Appendix. Abbreviations

F1+2: Prothrombin fragment 1+2
FDP: Fibrin degradation products
HUVEC: Human umbilical vein endothelial cells
MoAb: Monoclonal antibody
PBMC: Peripheral blood mononuclear cells
TNF-α: Tumor necrosis factor-alpha
VWF: von Willebrand factor

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1: American Journal of Cardiology, 1991, 68(7):B36-B50

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Thrombolytic therapy with urokinase reduces increased circulating endothelial adhesion molecules in acute myocardial infarction

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Abstract. The aim was to investigate circulating E-selectin and Intercellular Adhesion Molecule-1 (ICAM-1) in acute myocardial infarction. Our study was carried out in 80 patients, 40 hospitalized for acute myocardial infarction (AMI), 20 suffering from chronic stable angina and 20 healthy control subjects. Samples of venous blood were taken from all patients at the moment of hospitalization and after 2, 4, 6, 8, 10, 12 and 24 hours from the thrombolytic treatment (AMI + urokinase) or conventional therapy (AMI + nitroglycerin), for the dosage of creatinine kinase (CK) and adhesion molecules. The CK was determined by means of a Hitachi 901 automatic analyser using an enzymatic method (reagents Boheringer-Biochemia, Germany). Soluble E-selectin (sE-selectin) and soluble ICAM-1 (sICAM-1) were measured in the serum using a specific immunoassay (British Biotechnology Products). The serum levels of Tumor Necrosis Factor (TNF-α) were evaluated using an immunoenzymatic assay to quantitate the serum levels of the cytokine (British Biotechnology Products). Patients with acute myocardial infarction (AMI) had increased serum levels of soluble E-selectin (sE-selectin; AMI + urokinase = 312 ± 20 ng/ml; AMI + nitroglycerin = 334 ± 15 ng/ml) and soluble ICAM-1 (sICAM-1; AMI + urokinase = 629 ± 30 ng/ml; AMI + nitroglycerin = 655 ± 25 ng/ml) compared to both patients with chronic angina (sE-selectin = 67 ± 10 ng/ml; sICAM-1 = 230 ± 20 ng/ml) and healthy control subjects (sE-selectin = 53 ± 15 ng/ml; sICAM-1 200 ± 16 ng/ml). Furthermore patients with acute myocardial infarction also had increased serum levels of Tumor Necrosis Factor (TNF-$\alpha = 309 \pm 10$ pg/ml; control subjects = 13 ± 5 pg/ml). Thrombolytic therapy with urokinase (1,000,000 IU as an intravenous bolus for 5 minutes, followed by an infusion of an additional 1,000,000 IU for the following two hours) succeeded in producing reperfusion and reduced the serum levels of sE-selectin (52 ± 13 ng/ml) and sICAM-1 (202 ± 31 ng/ml). In contrast patients not eligible for thrombolytic therapy and therefore treated with conventional therapy (a continuous i.v. infusion of nitroglycerin at the dose of 50 mg/die) did not show any significant reduction in both sE-selectin and sICAM-1 throughout the study. Our results confirm previous experimental data and indicate that adhesion mechanisms supporting leukocyte-endothelium interaction may also be operative in human acute myocardial infarction.

Key words: E-selectin – Intercellular adhesion molecule 1 – Myocardial infarction – Leukocytes – Endothelium

Introduction

A large body of evidence has suggested the involvement of an inflammatory response in the pathophysiology of myocardial ischaemia-reperfusion injury [1]. Leukocyte accumulation in the myocardium may amplify tissue damage by producing cell activation of the myocytes and by releasing deleterious substances such as leukotrienes [2], thromboxane A_2 [3], oxygen free radicals [4] and platelet activating factor [5].

Adhesion molecules are considered to play a pivotal role in the localization and development of an inflammatory reaction. E-selectin is synthesized by the endothelium following different priming stimuli [6]. This molecule belongs to the selectin family and it has a molecular weight of 115 kDa [7]. The binding molecules for E-selectin in neutrophils and monocytes are the L-selectin ligands or Sialyl-Lewis^X [8]. Intercellular adhesion molecule 1 (ICAM-1) is an adhesion molecule normally expressed at a low basal level on endothelial cells, but its expression can be enhanced by various inflammatory mediators such as IL-1 and TNF [9]. Structurally ICAM-1 is a member of the Ig supergene family with five extracellular IG-like domains, a single transmembrane region and a short cytoplasmic tail [10]. It is a ligand for at least two members of the CD18 family of leukocyte

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adhesion molecules: LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) [11, 12].

It has been shown that a passive immunization with specific antibodies raised against both E-selectin and ICAM-1 reduces infarct size and myocardial leukocyte accumulation in an experimental model of myocardial ischaemia [13, 14].

Experimental evidence has also shown that soluble isoforms of these adhesion structures can be found in the circulation, thus indicating that the levels of soluble adhesion molecules may be useful monitors of disease activity and that they may have physiological effects.

We therefore investigated the serum levels of soluble E-selectin and ICAM-1 in patients with acute myocardial infarction.

Materials and methods

80 patients were studied, 40 hospitalized for acute myocardial infarction (AMI), 20 suffering from chronic stable angina (CHD) and 20 healthy normal control subjects (N).

The 40 patients with acute myocardial infarction were divided into two groups: a group of patients (n = 20) eligible for thrombolytic therapy and another group of patients (n = 20) that, because of the presence of exclusion criteria (previous coronary bypass operation, peptic ulcer disease, traumas or surgical operations in the last month, anticoagulant therapy), underwent a conventional treatment with nitroglycerin (a continuous intravenous infusion at the dose of 50 mg/die together with morphine hydrochloridum at the dose of 5 mg/die/i.v.)

Of the 20 patients hospitalized for a first event of AMI and treated with urokinase, 18 were male and 2 were female, with an age between 31 and 69 years (average age 54.3 ± 5.4 years).

In 12 cases the AMI was anterior and in 8 cases it was inferior. The interval of time between the onset of the first symptoms and hospitalization varied from 70 to 190 minutes (average 139.4 ± 36.7 minutes) and all patients were treated within 4 hours from the onset of the symptoms.

Of the 20 patients hospitalized for a first event of AMI and treated with conventional therapy, 12 were male and 8 were female, with an age between 35 and 72 years (average age 66.7 ± 6.3 years).

In 13 cases the AMI was anterior and in 7 cases it was inferior. The interval of time between the onset of the first symptoms and hospitalization varied from 80 to 220 minutes (average 151.4 ± 41.9 minutes) and all patients were treated within 4 hours from the onset of the symptoms.

The diagnosis of AMI was made on the basis of the presence of the characteristic precordial pain for more than 30 minutes not sensitive to the sublingual nitroglycerin, and the elevation in the ST segment in 2 or more contiguous precordial derivations (AMI anterior) or in 2 or 3 inferior derivations (II, III, aVF) (AMI inferior), the increase >100 U/l of the creatine kinase and >20 U/l of the isoenzyme MB of the CK.

Criteria of exclusion were the presence of a left bundle branch block, the depression of the ST segment or the inversion of the T-wave (non Q-wave infarction).

None of the patients showed, either at the moment of hospitalization or in the last month, existing infectious episodes and the urine test did not show signs of infections in progress. A cultural exam of the urine was performed anyway in all patients to confirm the absence of infectious processes of the genitourinary tract. None of the patients showed respiratory pathologies of the asthmatic type or autoimmune diseases.

Samples of venous blood were taken from all patients at the moment of hospitalization and after 2, 4, 6, 8, 10, 12 and 24 hours from the thrombolytic or conventional treatment, for the dosage of creatine kinase (CK) and soluble adhesion molecules.

The CK was determined by means of a Hitachi 901 automatic analyser using an enzymatic method (reagents Boheringer-Biochemia, Germany).

Soluble E-selectin (sE-selectin) and soluble ICAM-1 (sICAM-1) were used in the serum using a specific immunoassay (British Biotechnology Products). Briefly, 100 µl of biotinylated antibody raised against human E-selectin or human ICAM-1 and 100 µl of serum were added to each well. After washing with buffer and decanting, 100 µl of streptavidin conjugated to horseradish peroxidase were added to each sample. The wells were then incubated for 1 h and after washing with buffer and decanting 100 µl of tetramethylbenzidine were put into each well. The wells were then incubated for 10 min at 37 °C and, finally 100 µl of an acid solution were added to each well. The optical density of each well was determined within 30 minutes using the microtiter plate reader set at 450 nm with a correction wavelength of 620 nm.

The serum levels of Tumor Necrosis Factor (TNF-α) were evaluated using an immunoenzymatic assay to quantitate the serum levels of the cytokine (British Biotechnology Products).

The 20 patients, 16 males and 4 females, with an average age of 56.9 ± 7.2 (range 41–68 years), suffering from chronic stable angina, showed crises of angina not only of effort but also at rest and the sample was taken in a phase of quiescence, at least 72 hours from the last anginal event.

All of the patients showed an anginal symptomatology for at least three months. They had following a cycloergometer effort test either angina or a decrease in the ST segment. The ST segment decreased at least 1 mm at 0.08 sec. from point J.

The healthy control subjects showed no sign of respiratory, cardiovascular, infective or autoimmune diseases.

All of the subjects included in the case-record, informed about the purposes and methods of the study, gave their consent and the research was conducted in observance of the principles of the declaration of Helsinki and successive revisions of Tokyo and Venice.

Data are presented as mean ± S.D. The difference between the means of different groups was evaluated with ANOVA followed by Bonferroni's test and was considered significant when $p < 0.05$.

Results

8 of the 20 AMI urokinase patients were hypertensive and were previously treated with antihypertensive drugs (six with dihydropyridinic calcium antagonists, two with ACE-inhibitor and hydrochlorothiazide), 12 were smokers and 9 showed elevated cholesterol values (>240 mg/dl) (mean values 239 ± 27 mg/dl) (Table 1).

On admission to the clinic those patients underwent thrombolytic therapy with urokinase (Ukidan, Serono) administered at the dose of 1,000,000 IU by venous bolus for 5 minutes, followed by further 1,000,000 IU by infusion for the following two hours.

325 mg of aspirin were administered to all patients at the beginning of the thrombolytic therapy, while heparin was administered at the end of the thrombolytic treatment, at the dose of 5,000 U by intravenous bolus, followed by a continuous infusion of 1,000 U/hour, adjusting the dose to maintain a partial activated thromboplastin time between 70 and 100 seconds.

As indicators that reperfusion occurred, the relief of an early peak (<12 hours) of CK, the reduction of at least 50% of the decrease in the ST segment 90–120 minutes after the beginning of the thrombolysis, the sudden reduction or disappearance of thoracic pain and the onset of arrhythmia 1–90 minutes after the beginning of the therapy were used.

Table 1. Clinical characteristics of patients on admission to the study.

	AMI + urokinase (n = 20)	AMI + nitroglycerin (n = 20)	CHD patients (n = 20)	Healthy subjects (n = 20)
Age (years)	54.3 ± 5.4	66.7 ± 6.3	56.9 ± 7.2	52.8 ± 8.6
Men (n)	18	12	16	14
BMI	28.3 ± 3.4	29.5 ± 7.5	27.2 ± 2.1	26.3 ± 1.9
Site of infarction				
Anterior	12	13		
Inferior	8	7		
Onset of infarct to treatment				
Treatment within 3 hours	14	13		
Treatment within 4 hours	6	7		
Creatine kinase				
Peak (IU)	1471 ± 624	1375 ± 230		
Time to peak (hours)	9.3 ± 2.1	22 ± 3.2		
SBP (mmHg)	139 ± 21	148 ± 13	143 ± 9	137 ± 8
DBP (mmHg)	86 ± 13	87 ± 6	85 ± 5	82 ± 6
HR (beats/min)	81 ± 14	83 ± 7	78 ± 5	76 ± 4
Smokers (n)	12	5	10	8
Hypertension (n)	8	4	8	0
Serum TNF-α (pg/ml)	339 ± 10*	297 ± 11*	24 ± 7	13 ± 5
Cholesterol levels (mg/dl)	239 ± 27*	241 ± 15*	223 ± 25	212 ± 19
Hypercholesterolemia (n)	9	5	4	3
Creatinine clearance (ml/min)	101 ± 16	103 ± 15		
sE-selectin (ng/ml)	312 ± 20*	334 ± 15*	67 ± 10	53 ± 15
sICAM-1 (ng/ml)	629 ± 30*	655 ± 25*	230 ± 20	200 ± 16

AMI: acute myocardial infarction, CHD: coronary heart disease, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, * $p < 0.02$ vs CHD patients or healthy subjects. Data are expressed as mean ± S.D.

Following such criteria reperfusion was obtained in all the treated patients. The utilization of such criteria at the end of the evaluation seemed to us more useful than the data obtained from the coronarographic investigation, carried out at an interval variable from 7 to 30 days in all patients, since the results could be distorted by late phenomena of spontaneous recanalization or of reocclusion.

In patients with myocardial infarction and treated with urokinase the CK peak was reached between the sixth and the twelfth hour (mean values of CK peak 9.3 ± 2.1 hours) (Table 1). In contrast AMI patients treated with conventional therapy did not show an early peak of CK (Table 1). Figure 1 shows also the time course of serum CK before and after thrombolytic or conventional therapy.

Not having a pre-infarction value of sE-selectin and sICAM-1 the data obtained basally in such patients after the acute ischaemic event were compared with those of 20 patients suffering from ischaemic heart disease (chronic stable angina) and of 20 healthy subjects used as a control group, whose clinical characteristics are reported in Table 1. Basal levels of sE-selectin and sICAM-1 were significantly increased in patients with myocardial infarction compared both to patients with ischaemic heart disease and healthy control subjects (Fig. 2). Patients with acute myocardial infarction also had increased serum levels of TNF-α (Table 1).

Patients with ischaemic heart disease and healthy subjects had overlapping levels of sE-selectin and sICAM-1 (Fig. 2). Thrombolytic treatment significantly and progressively reduced the serum levels of the adhesion molecules (Figs. 3–4). In contrast AMI patients treated with conventional therapy did not show significant change in the soluble levels of both E-selectin and ICAM-1 throughout the study (Figs. 3–4).

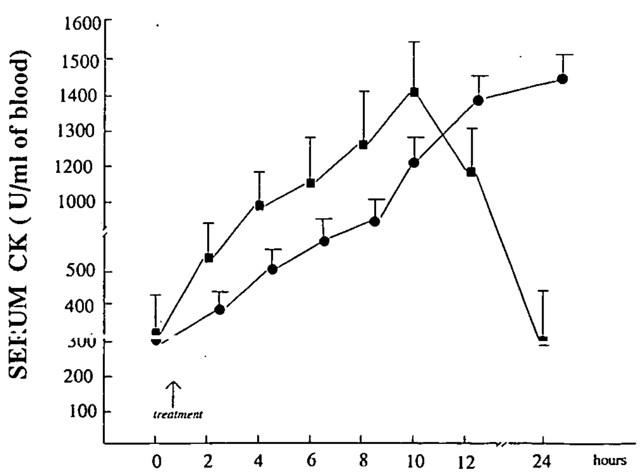

Fig. 1. Time course of serum CK before and after thrombolytic therapy or nitroglycerin treatment in patients with acute myocardial infarction (AMI). Each point represents mean ± S.D. from 20 patients. Closed squares = AMI + urokinase; Closed circles = AMI + nitroglycerin.

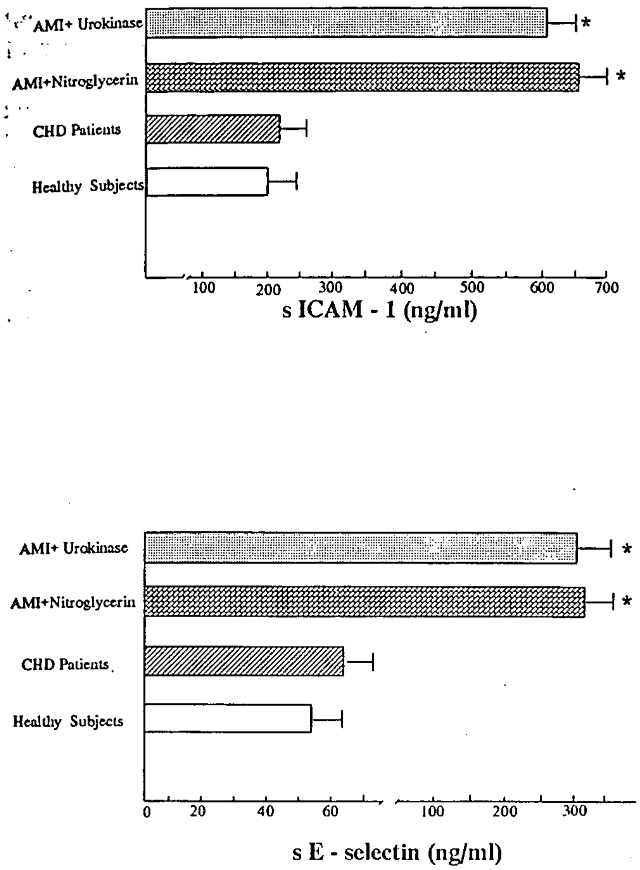

Fig. 2. Basal levels of circulating E-Selectin (sE-Selectin) and ICAM-1 (sICAM) in patients with acute myocardial infarction (AMI) later treated with urokinase (AMI + urokinase) or nitroglycerin (AMI + nitroglycerin), in patients with ischaemic heart disease (CHD) and healthy control subjects. Each point represents mean ± S.D. from 20 patients. $P < 0.001$ vs CHD patients or healthy subjects.

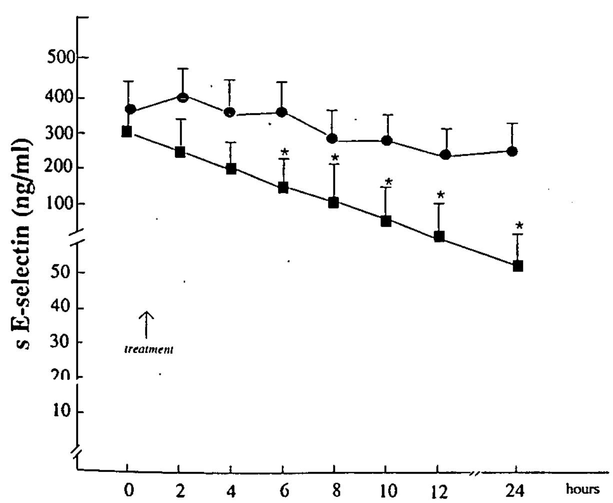

Fig. 3. Time course of circulating E-Selectin before and after thrombolytic therapy or nitroglycerin treatment in patients with acute myocardial infarction (AMI). Each point represents mean ± S.D. from 20 patients. * $P < 0.05$ vs AMI + nitroglycerin. Closed squares = AMI + urokinase; Closed circles = AMI + nitroglycerin.

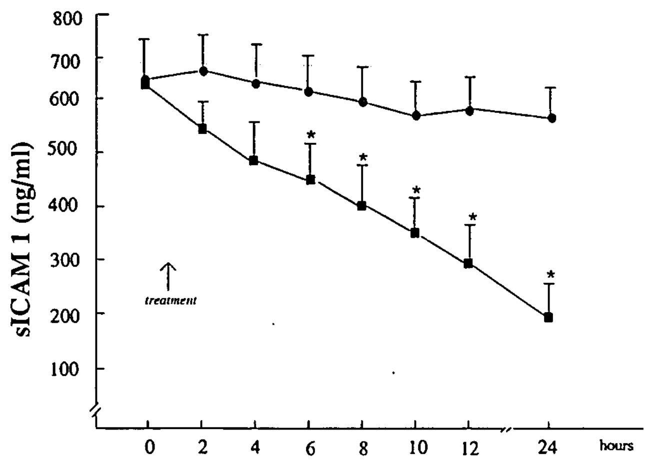

Fig. 4. Time course of circulating ICAM-1 before and after thrombolytic therapy or nitroglycerin treatment in patients with acute myocardial infarction (AMI). Each point represents mean ± S.D. from 20 patients. * $P < 0.05$ vs AMI + nitroglycerin. Closed squares = AMI + urokinase; Closed circles = AMI + nitroglycerin.

Discussion

A major factor involved in leukocyte recruitment into inflammatory tissues is thought to be the expression on vascular endothelial cells of cytokine inducible adhesion molecules for leukocytes [15, 16, 17]. The two adhesion molecules that have been characterized most fully are ICAM-1 and E-selectin, previously called endothelial adhesion molecule 1 [18]. ICAM-1 is a single chain glycoprotein of the IG supergene family which is present on unstimulated endothelial cells and on a variety of other cell types including activated fibroblasts, macrophages, and lymphocytes [18]. The surface expression of ICAM-1 is up-regulated on endothelial cells by IL-1, TNF, LPS or IFNΓ [19]. ICAM-1 is a ligand for the leukocyte integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) and is likely to be involved in the adhesion of both neutrophils and lymphocytes to cytokine activated endothelium. In contrast to ICAM-1, E-selectin is found only on activated endothelial cells and its expression is induced by IL-1 and TNF-α [19].

Previous findings have indicated that a passive immunization with specific antibodies raised against both E-selectin and ICAM-1 reduces infarct size and myocardial leukocyte accumulation in experimental myocardial ischaemia reperfusion injury. These results prompted us to study E-selectin and ICAM-1 in human acute myocardial infarction.

Experimental studies have identified a circulating form of both E-selectin [20] and ICAM-1 [21]. Elevated levels of sE-selectin have been reported in patients with diabetes, polyarteritis nodosoum, giant cell arteritis and scleroderma. sE-selectin showed also a marked increase in septic patients [22] and in hypertension [23].

A number of studies have already been published suggesting that circulating ICAM-1 is elevated in inflammation, infection and cancers [22]. So far, however, no study has been carried out in patients with myocardial infarction.

Therefore we measured the levels of both sE-selectin and sICAM-1 in patients with acute myocardial infarction.

Our results suggest that patients with acute myocardial infarction show elevated blood levels of both sE-selectin and sICAM-1 compared either to patients suffering from chronic stable angina (studied in a phase of quiescence) or healthy normal subjects. Furthermore, thrombolytic therapy significantly reduced the serum levels of the adhesion molecules. These observations, taken together, would suggest that only an acute ischaemic insult is able to produce a widespread activation of endothelium-leukocyte interaction. Indeed our data confirm previous data showing that soluble form of P-selectin, another adhesion molecule expressed by endothelial cells and platelets, is increased in acute myocardial infarction [24].

In contrast, the presence of a chronic ischaemic pathology such as that of the patients suffering from chronic stable angina does not cause the serum levels of soluble adhesion molecules to increase. However this latter result is, at least in part, in disagreement with the data reported by Blann and McCollum [25]. In fact they found an increase in sICAM-1 in patients suffering from coronary angina. This discrepancy might be due either to a different disease stage or, alternatively, to the fact that our patients were studied in a phase or quiescence.

It has been suggested that TNF-α induces "in vitro" the expression of both E-selectin and ICAM-1 [26]. We have previously shown that the serum levels of TNF-α are significantly increased in rats subjected to experimental myocardial ischaemia-reperfusion injury [27]. In agreement with these data our patients suffering from acute myocardial infarction had increased serum levels of TNF-α. Therefore it could be hypothesized that also in human myocardial infarction TNF-α induces the expression of both E-selectin and ICAM-1. Some patients with myocardial infarction had also increased serum levels of cholesterol: however at the present there is no scientific evidence supporting the hypothesis that hypercholesterolemia may cause the levels of either of the two soluble adhesion molecule or of TNF-α to increase.

An important finding of the present paper is the evidence that the thrombolytic therapy, which succeeded in producing reperfusion, progressively reduced the serum levels of circulating adhesion molecules. In contrast patients treated with conventional therapy had likely no early recanalization and showed no significant decrease in the soluble levels of E-selectin and ICAM-1 throughout the study.

This would indicate that the acute ischaemic event, likely through the release of several mediators such as inflammatory cytokines, platelet activating factor or eicosanoids, is responsible for the enhanced serum levels of both sE-selectin and sICAM-1. Indeed the data obtained from the measurement of soluble adhesion molecules may be differently interpreted. They may represent a marker of inflammation and, as in the case of sE-selectin, the increased serum levels would indicate endothelium activation or damage. Alternatively, circulating adhesion molecules might have physiological effects such as the capacity of competing in the cell–cell adhesion mechanisms, an event that would limit in myocardial ischaemia the deleterious accumulation of leukocytes in the endothelium and in the ischaemic tissues. However the scientific significance of monitoring levels of adhesion molecules is still far from being completely understood and only further studies will clarify this point.

In conclusion we have shown that patients with acute myocardial infarction have elevated serum levels of soluble endothelial adhesion molecules, thus indicating that therapeutical approaches aimed at blocking cell–cell interaction may represent a new avenue to the treatment of myocardial infarction.

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Thanks!

ACTIVATION OF COAGULATION AFTER ADMINISTRATION OF TUMOR NECROSIS FACTOR TO NORMAL SUBJECTS

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Abstract Tumor necrosis factor has been implicated in the activation of blood coagulation in septicemia, a condition commonly associated with intravascular coagulation and disturbances of hemostasis. To evaluate the early dynamics and the route of the in vivo coagulative response to tumor necrosis factor, we performed a controlled study in six healthy men, monitoring the activation of the common and intrinsic pathways of coagulation with highly sensitive and specific radioimmunoassays.

Recombinant human tumor necrosis factor, administered as an intravenous bolus injection (50 μg per square meter of body-surface area), induced an early and short-lived rise in circulating levels of the activation peptide of factor X, reaching maximal values after 30 to 45 minutes (mean ±SEM increase after 45 minutes, 34.2±18.2 percent; tumor necrosis factor vs. saline, P = 0.015). This was followed by a gradual and prolonged increase in the plasma concentration of the prothrombin fragment F_{1+2}, peaking after four to five hours (mean increase after five hours, 348.0±144.8 percent; tumor necrosis factor vs. saline, P<0.0001). These findings signify the formation of factor Xa (activated factor X) and the activation of prothrombin. Activation of the intrinsic pathway could not be detected by a series of measurements of the plasma levels of factor XII, prekallikrein, factor XIIa–C1 inhibitor complexes, kallikrein–C1 inhibitor complexes, and the activation peptide of factor IX. The delay between the maximal activation of factor X and that of prothrombin amounted to several hours, indicating that neutralization of factor Xa activity was slow.

We conclude that a single injection of tumor necrosis factor elicits a rapid and sustained activation of the common pathway of coagulation, probably induced through the extrinsic route. Our results suggest that tumor necrosis factor could play an important part in the early activation of the hemostatic mechanism in septicemia. (N Engl J Med 1990; 322:1622-7.)

SEPTICEMIA is frequently associated with disturbances of hemostatic balance. Disseminated intravascular coagulation, with widespread depositions of fibrin in the microvasculature, is commonly found in septic shock and is closely linked to the development of multiple organ failure.[1] The mechanism by which the clotting cascade is activated during septicemia is incompletely understood.

Recently, it has become apparent that the cytokine known as tumor necrosis factor has a pivotal role in the initiation of the septic syndrome. Tumor necrosis factor is secreted by monocytes and macrophages in response to various stimuli, of which endotoxins, derived from gram-negative bacteria, are the most potent.[2,3] Systemic release of tumor necrosis factor occurs soon after the injection of endotoxin in healthy volunteers,[4] and high levels of the factor have been detected in patients with sepsis.[5,6] In laboratory animals, recombinant tumor necrosis factor induces the septic syndrome,[7,8] whereas passive immunization against tumor necrosis factor prevents death in experimental models of sepsis.[9,10]

In cultured endothelial cells, tumor necrosis factor exerts a net procoagulant effect by enhancing the expression of tissue factor[11-13] and inhibiting the fibrinolytic response by suppressing the release of tissue-type plasminogen activator and inducing the secretion of plasminogen activator inhibitor Type I.[14-16] Moreover, the activation of protein C becomes impaired by down-regulation of thrombomodulin.[11,17,18] The infusion of high doses of recombinant tumor necrosis factor in dogs results in microvascular thrombosis.[8] In patients with meningococcal sepsis, the plasma levels of tumor necrosis factor are proportional to the extent of intravascular coagulation.[6] Hence, both in vitro and in vivo investigations have suggested that tumor necrosis factor is an important mediator of the activation of coagulation in septicemia.

In recent years tumor necrosis factor has been evaluated as an antineoplastic agent, given in low doses to patients with metastatic cancer. Bauer et al. confirmed the procoagulant effect of tumor necrosis factor in such patients by using highly sensitive and specific radioimmunoassays that permit the detection of in vivo activation of the hemostatic mechanism at the subnanomolar level (i.e., detection of the prothrombin fragment F_{1+2} and fibrinopeptide A).[19] The early events of the coagulative response could not be determined, however, since the first coagulation studies were performed three hours after the start of a continuous infusion of tumor necrosis factor. Moreover, a procoagulant state already existed before treatment with tumor necrosis factor, as indicated by elevated plasma levels of F_{1+2} and fibrinopeptide A at base line.[19] The aim of the present study was to investigate the early dynamics and route of coagulation activation after the administration of re-

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combinant tumor necrosis factor. Therefore, we performed a controlled study in six healthy male subjects, sequentially measuring indexes of the activation of the common and intrinsic pathways of coagulation.

METHODS

The study was approved by the institutional research and ethics committees of the Academic Medical Center, University of Amsterdam, and written informed consent was obtained from all subjects. All subjects were admitted to the Metabolic Research Ward.

Study Design

Six healthy men 27 to 33 years of age volunteered to participate in the study. None had abnormalities on physical examination or routine laboratory investigation. They did not use medications and had had no febrile illness in the month before the study. The study periods were 12 hours in length, starting at 7:30 a.m. The subjects fasted overnight until the end of each study period. Each subject was studied on two occasions at least three weeks apart. In one study period, a bolus intravenous injection of recombinant human tumor necrosis factor (50 μg per square meter of body-surface area) dissolved in 10 ml of isotonic saline was given; in the other period an equivalent volume of isotonic saline was administered. The order in which the two injections were given was determined randomly.

Mean arterial blood pressure and pulse rate were measured at 15-minute intervals with a Dinamap monitor (Critikon, Tampa). Temperature was recorded continuously by means of a rectal cannula (Hewlett-Packard, Boeblingen, Federal Republic of Germany).

Recombinant human tumor necrosis factor was kindly provided by Boehringer-Ingelheim (Ingelheim am Rhein, Federal Republic of Germany). It was more than 99 percent pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and contained less than 10 ng of endotoxin per milligram of protein, as tested by the limulus amebocyte lysate test.

Blood Collection

Venous blood samples were obtained by separate venipunctures, with use of 19-gauge butterfly needles, directly before the injection of recombinant tumor necrosis factor or isotonic saline and 15, 30, and 45 minutes and 1, 2, 3, 4, 5, 6, and 12 hours thereafter.

Blood for the measurement of factor IX activation peptide, factor X activation peptide, and F_{1+2} was collected in plastic syringes loaded with the following anticoagulant: 38 mM citric acid, 75 mM sodium citrate, 136 mM dextrose, 6 mM EDTA, 6 mM adenosine, and 25 U of heparin per milliliter. The ratio of anticoagulant to blood was 0.2:1.0 (vol/vol). After collection of the blood samples, plasma was obtained by centrifugation at 4°C for 30 minutes at 1600×g and stored at −70°C before measurement. Blood for the measurement of fibrinogen was collected in tubes loaded with 32 g of trisodium citrate dihydrate per liter (1 to 9 ml of blood) and centrifuged at 1600×g for 20 minutes at room temperature. The plasma samples were stored at −70°C until analyzed.

Blood for the determination of factor XII, prekallikrein, factor XIIa-C1 inhibitor complexes, and kallikrein-C1 inhibitor complexes was collected in siliconized Vacutainer tubes (Becton Dickinson, Plymouth, England), to which EDTA (10 mM) and Polybrene (0.05 percent, wt/vol) were added to prevent any in vitro activation of the contact system.[20] The tubes were centrifuged at room temperature for 10 minutes at 1300×g, and the plasma was aliquoted and stored in polystyrene tubes at −70°C until the tests were performed. Blood for the determination of platelet counts was collected in tubes loaded with EDTA tripotassium and analyzed immediately. Tumor necrosis factor was measured in serum samples obtained immediately before the injection of recombinant tumor necrosis factor and saline and 5, 10, 15, 30, 45, 60, 120, 180, and 240 minutes thereafter. The serum samples were frozen immediately and kept frozen until assayed.

Assays

The plasma levels of factor IX activation peptide, factor X activation peptide, F_{1+2}, factor XII, prekallikrein, factor XIIa-C1 inhibitor complexes, and kallikrein-C1 inhibitor complexes were determined by radioimmunoassays as described elsewhere.[21-25] Plasma fibrinogen concentrations were measured with a turbidimetric method (ChromoTimeSystem, Behringwerke, Marburg, Federal Republic of Germany).[26] The plasma concentrations of factor IX activation peptide and factor X activation peptide are given in picomoles per liter, those of F_{1+2} in nanomoles per liter, and the plasma fibrinogen values in grams per liter. Plasma levels of factor XII and prekallikrein are expressed as units per milliliter of plasma, by reference to pooled plasma from healthy donors that contains one unit of both factor XII and prekallikrein per milliliter. The plasma values of factor XIIa-C1 inhibitor complexes and kallikrein-C1 inhibitor complexes are expressed as units per milliliter of plasma, by reference to dextran sulfate plasma, pooled from healthy donors, that contains one unit of both factor XIIa-C1 inhibitor complexes and kallikrein-C1 inhibitor complexes per milliliter.[25] The radioimmunoassays for these C1-inhibitor complexes can detect the activation of 0.05 percent of plasma factor XII or prekallikrein.[25] All samples obtained for measurement of the activation of the common and intrinsic pathway of coagulation were assayed in one to four runs. Each run contained both samples from the subjects given saline and samples from subjects given tumor necrosis factor, and care was taken that all the samples from one subject were assayed in the same run. The interassay coefficients of variation of the assays used were as follows: factor IX activation peptide, 12 percent[21]; factor X activation peptide, 12 percent[22]; F_{1+2}, 8 percent[19]; factor XII and prekallikrein, <10 percent[25]; factor XIIa-C1 inhibitor complexes and kallikrein-C1 inhibitor complexes, <9 percent[25]; and fibrinogen, <9 percent.[26]

Platelet counts were determined with the use of a flow cytometer (Technicon H1 system, Technicon Instruments, Tarrytown, N.Y.). Serum levels of tumor necrosis factor were determined by immunoradiometric assay (Medgenix, Fleurus, Belgium). Polypropylene tubes were coated with a combination of monoclonal antibodies to recombinant tumor necrosis factor that recognize distinct epitopes of tumor necrosis factor. These tubes were incubated overnight with a mixture of the sample to be tested and anti-tumor-necrosis-factor antibody labeled with iodine-125. After decantation, the bound fraction was counted in a gamma counter, and the level of tumor necrosis factor was expressed in picograms per milliliter in relation to a standard binding curve for recombinant human tumor necrosis factor.

Statistical Analysis

Values are given as means ±SEM. Differences in results between the tumor necrosis factor and saline experiments were tested by analysis of variance and Student's paired t-test, as indicated. A P value <0.05 was considered to represent a significant difference.

RESULTS

Clinical Features

Tumor necrosis factor induced severe headache and nausea in all subjects, accompanied by vomiting in three subjects. The symptoms started as early as 10 minutes after the injection and lasted several hours. No significant changes in hemodynamic indexes were observed, and all subjects had recovered fully by the end of the experiment. Each subject had a rise in body temperature, preceded by chills. Peak temperatures (38.7±0.2°C) were reached after three hours. N ne of

these changes were noted during the control period, in which saline was administered.

Common Pathway of Coagulation

The activation of the common pathway of coagulation was monitored by the determination of plasma levels of factor X activation peptide, the prothrombin fragment F_{1+2}, and fibrinogen. The base-line values for these indexes of coagulation activation were similar in both study periods. The plasma levels of factor X activation peptide and F_{1+2} remained unchanged during the control period. As compared with saline, tumor necrosis factor induced an early and transient increase in plasma concentrations of factor X activation peptide ($P = 0.015$ by analysis of variance; Fig. 1). Maximal plasma levels of factor X activation peptide were reached 30 to 45 minutes after the injection of tumor necrosis factor (from 67.6±8.1 pmol per liter at base line to 86.6±9.1 pmol per liter at 45 minutes; mean increase, 34.2±18.2 percent). The administration of tumor necrosis factor was also associated with a significant increase in levels of F_{1+2}, as compared with saline ($P<0.0001$ by analysis of variance) (Fig. 1). This more gradual increase became apparent after one hour. Peak plasma levels of F_{1+2} were observed after four to five hours (from 1.14±0.30 nmol per liter at base line to 3.66±0.77 nmol per liter at five hours; mean increase, 348.0±144.8 percent). Thereafter, the plasma levels of F_{1+2} decreased gradually but were still elevated 6 to 12 hours after the injection of tumor necrosis factor. The plasma concentrations of fibrinogen did not change after the injection of either tumor necrosis factor or saline (data not shown).

Intrinsic Pathway of Coagulation

During the entire observation period, the circulating levels of factor XIIa–C1 inhibitor complexes and kallikrein–C1 inhibitor complexes, both of which reflect activation of the contact system, as well as the plasma values of factor XII and prekallikrein, the zymogen proteins of the contact system, remained within the normal range after the administration of both tumor necrosis factor and saline. In addition, during the 12 hours of the study, plasma levels of factor IX activation peptide, a measure of in vivo activation of factor IX, were not significantly affected by tumor necrosis factor as compared with saline.

Table 1 shows the results for the indexes of intrinsic-pathway activation during the first 45 minutes after the administration of tumor necrosis factor, in which factor X activation was maximal.

Platelet Counts

The platelet counts showed no significant changes after the injection of either tumor necrosis factor or saline (data not shown).

Serum Levels of Tumor Necrosis Factor

Tumor necrosis factor was not detectable in serum obtained before the injections of tumor necrosis factor or saline or in the serum samples collected during the control period. After the injection of tumor necrosis factor, the highest serum level of this factor was measured in the first blood sample, taken after five

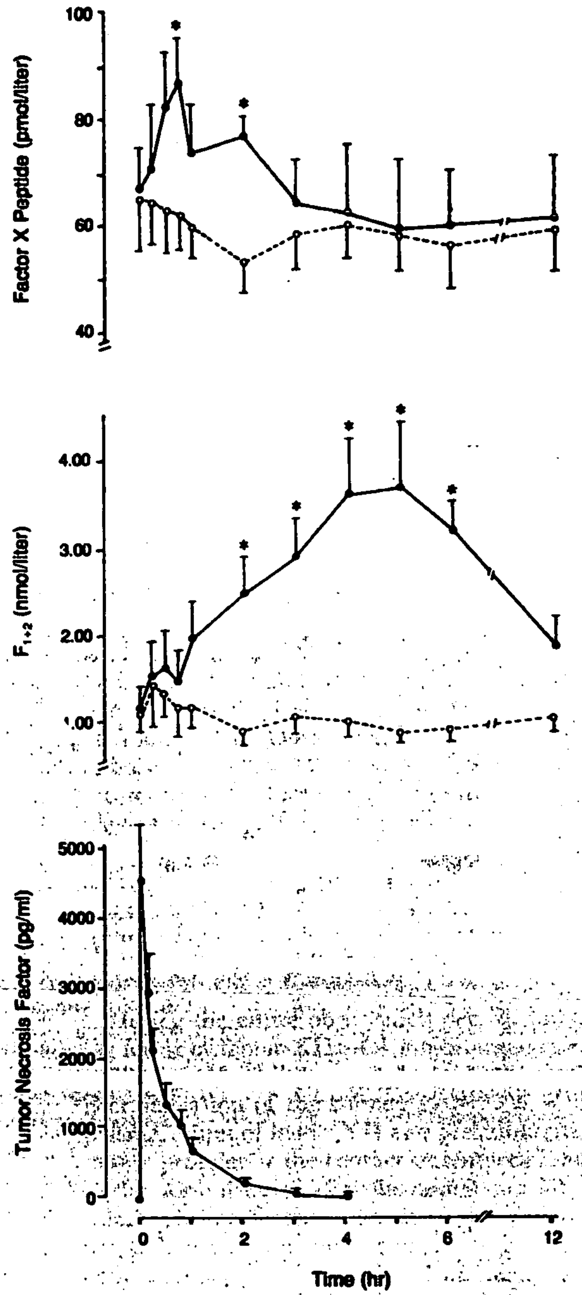

Figure 1. Mean (±SEM) Plasma Levels of Factor X Activation Peptide and the Prothrombin Fragment F_{1+2} and Serum Levels of Tumor Necrosis Factor after Intravenous Bolus Injections of Recombinant Human Tumor Necrosis Factor (50 μg per Square Meter; Solid Circles) or an Equivalent Volume of Isotonic Saline (Open Circles).

Asterisks indicate statistical significance for the comparison of tumor necrosis factor with saline ($P<0.05$ by Student's paired t-test). No circulating tumor necrosis factor was detected in the control period.

Table 1. Indexes of Activation of the Intrinsic Pathway of Coagulation during the First 45 Minutes after the Administration of Recombinant Human Tumor Necrosis Factor.*

Minutes after Injection	Factor XII	Factor XIIa–C1 Inhibitor Complexes	Prekallikrein	Kallikrein–C1 Inhibitor Complexes	Factor IX Activation Peptide
	U/ml	mU/ml	U/ml	mU/ml	pmol/liter
0	0.89±0.08	13±2	0.72±0.04	73±10	236.5±47.1
15	0.91±0.09	15±2	0.72±0.06	83±15	207.9±42.2
30	0.93±0.10	12±2	0.72±0.05	73±12	212.8±40.1
45	0.87±0.10	14±3	0.74±0.06	88±17	236.9±50.5

*Values are means ±SEM.

minutes (4261±785 pg per milliliter). Thereafter, serum levels of tumor necrosis factor decreased rapidly (Fig. 1).

Discussion

Tumor necrosis factor is a polypeptide hormone with a wide range of biologic activities. Its role as a crucial mediator of septic shock has been well established. Several studies suggest that tumor necrosis factor may also be implicated in the induction of coagulation activation seen in septicemia. The recent development of a unique set of highly sensitive and specific radioimmunoassays that monitor the transitions of coagulation-system zymogens to serine proteinases allowed us to study the dynamics of the in vivo procoagulant effect of low-dose tumor necrosis factor (50 μg per square meter) at the subclinical level. It was demonstrated that a single intravenous bolus injection of recombinant tumor necrosis factor induced a rapid activation of the common pathway of the coagulation system in healthy subjects, as indicated by a brief increase in plasma levels of factor X activation peptide, peaking after 30 to 45 minutes, followed by a gradual increase in the plasma levels of the prothrombin fragment F_{1+2}, which remained elevated for 6 to 12 hours.

Factor X activation peptide is liberated from factor X during the proteolytic cleavage of this zymogen by factor IXa or the factor VIIa–tissue factor complex,[22] representing activation by the intrinsic and extrinsic routes, respectively. The F_{1+2} fragment is released from prothrombin during its conversion to thrombin.[23,24] Therefore, these peptides directly monitor the in vivo activation of factor X and prothrombin. Since tumor necrosis factor does not affect the metabolic behavior of F_{1+2},[19] the elevation in the level of this fragment must have resulted from the excessive activity of factor Xa on prothrombin. Although the influence of tumor necrosis factor on the turnover of factor X activation peptide has not been examined, it is likely that the increase in this activation peptide was also caused by enhanced production.

Historically, common-pathway activation is supposed to proceed by either the intrinsic or extrinsic route. The intrinsic route is initiated by the activation of the contact system, during which the zymogens factor XII and prekallikrein are converted to factor XIIa and kallikrein, respectively.[27] These enzymes are rapidly inactivated by circulating proteinase inhibitors, of which C1 inhibitor is the most important. In patients with sepsis, decreased plasma levels of factor XII and prekallikrein[25,27] and increased levels of factor XIIa–C1 inhibitor complexes and kallikrein–C1 inhibitor complexes[25] have been reported, and they are interpreted to indicate contact activation. In the present study, the plasma levels of these zymogens and proteinase-inhibitor complexes remained within the normal range after the injection of tumor necrosis factor, indicating that the contact system was not stimulated. The absence of intrinsic-route activation was further supported by the observation that the plasma levels of factor IX activation peptide, a fragment formed during the conversion of factor IX to factor IXa and thus indicative of in vivo activation of factor IX,[21] were not significantly affected by the administration of tumor necrosis factor. The noninvolvement of the contact system in the activation of coagulation after the low-dose injection of tumor necrosis factor may indicate that although there is evidence that the contact system is activated in the course of septicemia, it is not required for the initiation of the coagulative response.

Since the intrinsic pathway was not activated in our experiment, the generation of factor Xa that we observed must have been the result of activation of the extrinsic route or alternative pathways. The extrinsic route is initiated by the expression of tissue-factor activity. Evidence for the in vivo induction of the extrinsic route in septicemia has been provided by the observation of increased tissue-factor activity in the monocytes of patients with meningococcal infection.[28] Under in vitro conditions, tumor necrosis factor can stimulate the synthesis of tissue factor in endothelial and mononuclear cells, but this effect becomes apparent only after several hours.[11-13,29] However, recent immunohistochemical studies have detected substantial expression of tissue factor in the vascular tunica adventitia, which is anatomically sequestered from blood.[30] The rapid generation of factor Xa observed in our study may have been the result of the exposure of this subendothelial tissue factor to plasma proteins, facilitated by increased vascular permeability induced by tumor necrosis factor.[31,32] The activation of the common pathway, however, could be the result of direct proteolytic cleavage of factor X by an alternative mechanism. It has been demonstrated that factor X can be rapidly bound to the adhesive receptor Mac-1 (a component of the CD11/18 complex) on stimulated monocytes and subsequently activated.[33] Tumor necrosis fact r elicits the expressi n of the CD11/18 complex,[34] but whether this enables factor X to be activated is currently unknown.

Peak plasma levels of F_{1+2} were reached tw to five

hours after the maximal plasma concentrations of factor X activation peptide, indicating a delay between the maximal activation of factor X and that of prothrombin. Given that the plasma half-lives of factor X activation peptide and F_{1+2} are relatively short (15 and 90 minutes, respectively[22,35]), our results indicate that the activation of prothrombin continued even though the generation of factor Xa had already returned to the levels observed before the administration of tumor necrosis factor. Since the injected tumor necrosis factor was cleared from the circulation rapidly (Fig. 1) and no activation of the intrinsic route was observed throughout the experiment, the sustained activation of prothrombin must have resulted from the early activation of factor X. Apparently, the activity of factor Xa was neutralized only slowly — an observation that challenges our current understanding of the inhibitory regulation of coagulation proteinases in vivo and that may be relevant to the pathophysiologic features of thrombotic diseases. In particular, the repeated or prolonged release of tumor necrosis factor into the circulation, as has been observed in patients with sepsis, may exert a cumulative effect in the procoagulant state. The previous report that factor Xa sequestered on the surface of a platelet or phospholipid in vitro cannot be inhibited by the antithrombin III–heparin complex[36] may explain this continuing formation of thrombin in the presence of the natural anticoagulant mechanisms.

Thrombocytopenia is a major feature of disseminated intravascular coagulation, believed to result from the increased consumption of platelets.[1] In patients with cancer, a decline in platelet counts has been reported after the systemic administration of tumor necrosis factor.[19,37,38] In contrast, we did not find significant changes in platelet counts after the injection of tumor necrosis factor. The reasons for this discrepancy are currently unknown, but they may be related to differences in the duration of the infusion of tumor necrosis factor or in the doses used, or to an altered susceptibility to tumor necrosis factor in patients with cancer.

This controlled study clearly shows that low-dose tumor necrosis factor induces a subclinical activation of the coagulation system, supporting the hypothesis that tumor necrosis factor is involved in the pathogenesis of hemostatic disorders associated with sepsis. Knowledge of the mechanisms responsible for the onset and maintenance of this activation may lead to the development of effective strategies for the treatment of patients with disseminated intravascular coagulation and multiple organ failure.

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A CONTROLLED TRIAL OF TRANSCUTANEOUS ELECTRICAL NERVE STIMULATION (TENS) AND EXERCISE FOR CHRONIC LOW BACK PAIN

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Abstract A number of treatments are widely prescribed for chronic back pain, but few have been rigorously evaluated. We examined the effectiveness of transcutaneous electrical nerve stimulation (TENS), a program of stretching exercises, or a combination of both for low back pain. Patients with chronic low back pain (median duration, 4.1 years) were randomly assigned to receive daily treatment with TENS (n = 36), sham TENS (n = 36), TENS plus a program of exercises (n = 37), or sham TENS plus exercises (n = 36).

After one month no clinically or statistically significant treatment effect of TENS was found on any of 11 indicators of outcome measuring pain, function, and back flexion; there was no interactive effect of TENS with exercise. Overall improvement in pain indicators was 47 percent with TENS and 42 percent with sham TENS (P not significant). The 95 percent confidence intervals for group differences excluded a major clinical benefit of TENS for most outcomes. By contrast, after one month patients in the exercise groups had significant improvement in self-rated pain scores, reduction in the frequency of pain, and greater levels of activity as compared with patients in the groups that did not exercise. The mean reported improvement in pain scores was 52 percent in the exercise groups and 37 percent in the nonexercise groups (P = 0.02). Two months after the active intervention, however, most patients had discontinued the exercises, and the initial improvements were gone.

We conclude that for patients with chronic low back pain, treatment with TENS is no more effective than treatment with a placebo, and TENS adds no apparent benefit to that of exercise alone. (N Engl J Med 1990; 322:1627-34.)

In the United States low back pain, often of a chronic nature, results in expenditures of $13 billion a year for medical care.[1-4] A number of simultaneous treatments are usually advocated for patients with chronic pain, but few of these treatments have ever been subjected to rigorous clinical evaluation.

Transcutaneous electrical nerve stimulation (TENS) is widely used in the management of chronic pain.[5] The use of conventional (high-frequency) TENS was originally based on the gate-control theory of pain,[6] which suggested that counterstimulation of the nervous system could modify the perception of pain. Later studies suggested that with low-frequency, high-amplitude ("acupuncture-like") stimulation, TENS could also raise endorphin levels in the spinal fluid.[7] Nationwide data on the use of TENS are unavailable, but in 1986 the Veterans Administration spent nearly $2 million on TENS units, and the labor costs for personnel to operate the device are high. TENS units are approved for payment by most third-party payers, including Medicare.

Despite its wide use and theoretical rationale, there is meager evidence from controlled clinical trials of the

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Tumor necrosis factor involvement in myocardial ischaemia-reperfusion injury

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The role of tumor necrosis factor-α was investigated in an anaesthetized rat model of coronary artery ligation (60 min) and reperfusion (MI/R). Sham-occluded rats (sham MI/R) were used as controls. Survival rate, myocardial necrosis, myocardial myeloperoxidase activity, serum creatinine kinase activity and serum and macrophage tumor necrosis factor-α were studied. Ischaemia-reperfusion injury significantly reduced survival rate (45%), produced marked myocardial injury, increased serum creatinine kinase activity and increased myocardial myeloperoxidase activity in the area-at-risk and in the necrotic area. Serum tumor necrosis factor-α was undetectable during the occlusion period, but increased significantly upon release of the coronary artery. At the end of reperfusion, macrophage tumor necrosis factor-α was also increased. Passive immunization with a hyperimmune serum containing antibodies against murine tumor necrosis factor-α significantly increased survival rate (80%), lowered myocardial necrosis, reduced the increase in serum creatinine kinase activity and decreased myeloperoxidase activity in the area-at-risk and in the necrotic area. These data are consistent with an involvement of tumor necrosis factor-α in myocardial ischaemia-reperfusion injury.

TNFα (tumor necrosis factor-α); Anti-tumor necrosis factor-α antibodies; Ischaemia-reperfusion injury

1. Introduction

Early reperfusion represents one of the most effective means of reducing myocardial damage in acute myocardial infarction. However, even under these conditions there is often marked cardiac injury (Lucchesi, 1990). Various sources of experimental evidence suggest that leukocytes may have a pivotal role in the phenomenon of myocardial reperfusion injury (Lucchesi, 1990; Dinerman and Mehta, 1990). During inflammation and ischaemic states circulating leukocytes are localized along the vessel wall where they adhere to the endothelial cell, cross the endothelial barrier through interendothelial cell junctions and accumulate in subcellular regions (Butcher, 1990; Osborn, 1990). Leukocytes can cause irreversible changes (cell death) in myocytes by releasing damaging and harmful substances such as oxygen free radicals (Downey, 1990), leukotrienes (Feuerstein, 1984), thromboxane A_2 (Lefer and Darius, 1987), platelet activating factor (Bracquet et al., 1987) and cytokines (Vane et al., 1990).

Endothelial cells, therefore, play an important role in the inflammatory and ischaemic response. In fact, they undergo significant functional changes that increase leukocyte adhesiveness to the endothelium. These include expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1), thus leading to the binding and extravasation of leukocytes, as well as to the release of soluble mediators (Harlan, 1987; Ziff, 1989). Many of these changes can be imitated in vitro by the addition of recombinant tumor necrosis factor-α to endothelial cell monolayers (Pober and Cotran, 1990; Mantovani and Dejana, 1989). In addition, local injection of tumor necrosis factor-α results in a rapid recruitment of leukocytes from the blood and this cytokine can be detected at sites of inflammatory lesions (Munro et al., 1989). Furthermore tumor necrosis factor-α causes endothelial dysfunction and a reduced formation and release of endothelial-dependent relaxing factor (EDRF) (Aoky et al., 1989). These studies predict an important role for such a cytokine in controlling the phenotype and function of

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endothelial and other inflammatory cells during inflammation and reperfusion injury.

In keeping with this hypothesis, it has been shown that tumor necrosis factor-α, apart from its role in endotoxin shock (Beutler and Cerami, 1987a), may also be an important mediator of non-septic ischaemic states. Evidence suggests that this cytokine is involved in bowel ischaemia-reperfusion injury (Squadrito et al., 1992) and in hepatic ischaemia-reperfusion injury (Colletti et al., 1990) and recently we have shown that tumor necrosis factor-α is present in the bloodstream of rats subjected to myocardial ischaemia-reperfusion injury (Caputi and Squadrito, 1992).

We therefore studied whether passive immunization with specific antibodies raised against murine tumor necrosis factor-α can protect against myocardial ischaemia-reperfusion injury.

2. Materials and methods

2.1. Ischaemia-reperfusion injury

Male normotensive Sprague-Dawley rats (Charles River, Italy) weighing 250–375 g were used. The animals were anaesthetized with sodium pentobarbitone (50 mg/kg i.p.) and the level of anaesthesia was maintained throughout the experiment by giving additional doses of the anaesthetic. A catheter (PE 50) was inserted into the common carotid artery to measure blood pressure and heart rate directly, as reported previously (Caputi et al., 1980). The jugular vein was also cannulated for drug or saline administration. The animals were instrumented for ECG lead II recordings. The trachea was cannulated and artificial respiration with room air was started at a frequency of 60 strokes/min with a tidal volume of 1 ml/100 g. The technique to produce myocardial ischaemia-reperfusion injury is similar to that described previously (Petty et al., 1991). An incision was made on the left side of the chest and the fourth intercostal space was exposed. Sutures were placed through the overlapping skin and muscles to permit rapid closure of the chest wall after the surgical procedures. The chest was then opened and the ribs were gently spread. The heart was quickly removed from the thoracic cavity, inverted, and a 4.0 silk ligature was placed under the left main coronary artery. The ligature was then either occluded for a period of 1 h, followed by 60 min of reperfusion (MI/R rats), or removed (sham MI/R rats). The heart was returned quickly to the thoracic cavity and the incision was closed by tying the previously placed sutures. Animals were injected i.v. with specific anti-tumor necrosis factor-α antibodies (2 mg/kg i.v.) dissolved in 0.3 ml of a phosphate-buffered solution at pH 7.4; control rats received a non-immune serum dissolved in the carrier vehicle 3 h before occlusion.

2.2. Myocardial tissue analysis

Myocardial injury was determined by the triphenyl tetrazolium chloride-Evans blue technique (Flameng et al., 1986). At the end of the 2 h experimental period the ligature around the left main coronary artery was tightened again; 2 ml of 5% Evans blue dye was injected into the jugular vein to stain the area of the myocardium perfused by the patent coronary arteries. The area-at-risk was therefore determined by negative staining. The atria, right ventricle and major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into 3-mm-thick sections parallel to the atrioventricular groove. The unstained portion of the myocardium (i.e. the area-at-risk) was separated from the stained portion (i.e. the area-not-at-risk). The unstained portion was again sliced into 1-mm-thick sections and incubated in a 0.1% solution of nitroblue tetrazolium stain in phosphate buffer at pH 7.4 at 37°C for 15 min to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium, which did not stain, was separated from the stained portion (i.e. the non-necrotic area-at-risk). Samples from all three portions of left ventricular cardiac tissue (i.e. non-ischaemic, ischaemic non-necrotic, ischaemic-necrotic) were weighed and stored at −70°C for subsequent assay of myeloperoxidase activity.

2.3. Creatinine kinase activity

At the end of the reperfusion period, blood was collected in polyethylene tubes. Samples were centrifuged at 2400 × *g* and 4°C for 15 min and the serum was removed for biochemical analysis. Creatinine kinase activity was measured using a previously described method (Rosalki, 1967).

2.4. Myeloperoxidase activity

The usefulness of measuring myeloperoxidase activity to assess leukocyte infiltration has been reported previously (Mullane et al., 1985; Lefer et al., 1991) and validated recently for rat cardiac tissue (Griswold et al., 1988). Briefly, myeloperoxidase activity was determined in all three portions of left ventricular cardiac tissue (i.e. non-ischaemic, ischaemic non-necrotic and ischaemic-necrotic) obtained, as described above, after the end of the reperfusion period, thereby permitting the simultaneous assessment of leukocyte infiltration and myocardial injury. The samples were first homogenized in a solution containing 20 mM of potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 U/ml of

a protease inhibitor (aprotinine) in proportions of 1:10 (w:v) and then centrifuged for 30 min at $20000 \times g$ at 4°C. The supernatants of each sample were then discarded and the pellet was immediately frozen on dry ice. Samples were frozen for one night before they were sonicated. After the samples had thawed, they were added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 50 mM potassium phosphate buffer (pH 6) containing 30 U/ml of protease inhibitor. Each sample was then sonicated for 1 min at intensity 2 and at a temperature of 4°C. After sonication the samples were allowed to cool on ice for approximately 30 min, and then centrifuged for 30 min at $40000 \times g$ at 4°C. An aliquot of the supernatant was incubated with 0.167 mg/ml O-dianisidine dihydrochloride (Sigma Chemical Co.) and 0.0010% H_2O_2, and the rate of change in absorbance was measured at 405 nm in a Micro-ELISA instrument. Myeloperoxidase activity is defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 25°C and is expressed in units per gram weight ($U \times 10^{-3}$) of tissue.

2.5. *Assay for tumor necrosis factor-α activity*

Blood samples were taken before occlusion, at the end of the occlusion period, at the start of reperfusion and 30 and 60 min after the start of reperfusion. Furthermore, at the end of reperfusion peritoneal macrophages were collected as described before by Altavilla et al. (1989). Killing of L929 mouse tumor cells was used to measure tumor necrosis factor-α in serum and in peritoneal macrophage supernatants on the basis of a standard assay (Altavilla et al., 1989). L929 cells in RPMI 1640 medium containing 5% foetal calf serum were seeded at 3×10^4 per well in 96-well microdilution plates and incubated overnight in an atmosphere of 5% of CO_2 in air. Briefly, peritoneal cells were diluted to 10^6/ml, plated on 35-mm-diameter plastic Petri dishes and allowed to adhere for 1 h at 37°C in a 5% CO_2 atmosphere. Dishes were carefully washed in order to eliminate non-adherent cells. Adherent cells were then incubated with RPMI for 2 h at 37°C in 5% CO_2; afterwards supernatants were collected. Serial 1:2 dilutions of serum and supernatants of peritoneal macrophages, were made up in the above medium containing 1.0 μg of actinomycin D per ml, and 100 μl volumes of each dilution were added to the wells. The next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol) and 0.1 ml of 1% sodium dodecyl sulphate was added to each well to solubilize the stained cells. The absorbance of each well was read at 490 nm with a model BT-100 Microelisa Autoreader. The percentage of cytotoxicity was calculated as $1-[(A490$ of sample$/A490$ of control$)] \times 100$. One tumor necrosis factor-α unit is defined as the amount of tumor necrosis factor-α giving 50% cytotoxicity. The tumor necrosis factor-α content in the samples was calculated by comparison with a calibration curve made with recombinant murine tumor necrosis factor-α (Nuclear Laser Medicine, Milan, Italy). To verify if the cytotoxicity tested was due to tumor necrosis factor-α or to other factor(s), we preincubated our samples for 2 h at 37°C with an excess of rabbit anti-recombinant murine tumor necrosis factor-α polyclonal antibodies (Nuclear Laser Medicine, Milan, Italy) or with control rabbit serum. Our results showed that cytotoxicity against L929 cells was completely neutralized by the rabbit anti-recombinant tumor necrosis factor-α polyclonal antibodies but not by the control rabbit serum.

2.6. *Cardiovascular measurements*

Mean arterial blood pressure (MAP) and heart rate (HR) were monitored throughout the experiment. The pressure rate index was calculated as the product of MAP and HR (Gobel et al., 1978) and is reported as mm Hg/beats per min $\times 10^{-3}$.

2.7. *Drug*

A highly specific polyclonal rabbit antiserum directed against murine tumor necrosis factor-α and containing 10^6 neutralizing units/mg was purchased from Nuclear Laser Medicine, Milan, Italy. This antiserum did not react with tumor necrosis factor-β or other factors. Control rats were injected with a non-immune rabbit serum dissolved in the carrier vehicle (0.3 ml of phosphate-buffered saline solution at pH 7.4). Neither the immune nor the non-immune serum contained endotoxin.

2.8. *Statistical analysis*

The difference between the means of two groups was evaluated with an ANOVA followed by Bonferroni's test and was considered significant when $P < 0.05$ (Squadrito et al., 1992). For survival data, statistical analysis was done with Fisher's exact probability test.

3. Results

3.1. *Survival*

The survival rate decreased after coronary reperfusion. Figure 1 summarizes the survival of sham-occluded or occluded and reperfused animals (sham MI/R or MI/R, respectively). There were 20 animals in each group. The survival rate at the end of reperfu-

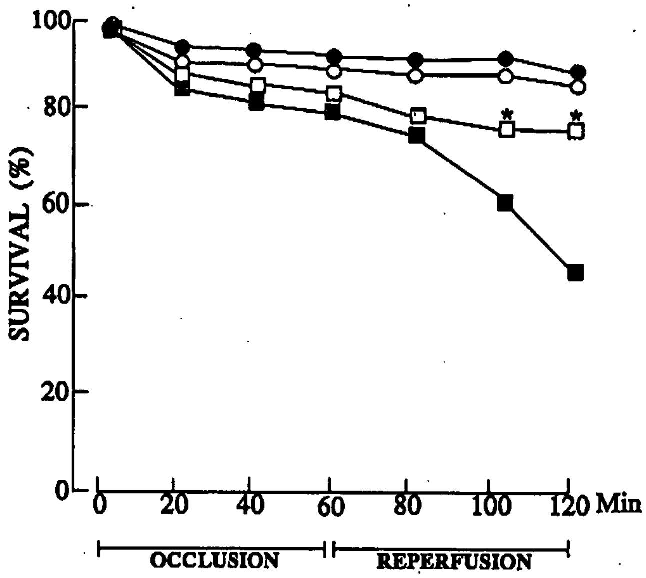

Fig. 1. Effects of passive immunization with specific antibodies against tumor necrosis factor-α on survival rate in rats subjected to myocardial ischaemia-reperfusion injury (MI/R). The antibodies (2 mg/kg i.v.) or a non-immune serum (0.3 ml/i.v. dissolved in a phosphate-buffered saline solution at pH 7.4) were injected 3 h before the coronary artery occlusion. * $P < 0.001$ vs. MI/R + non-immune serum. Sham MI/R + non-immune serum (●); sham MI/R + antibodies (○); MI/R + non-immune serum (■); MI/R + antibodies (□).

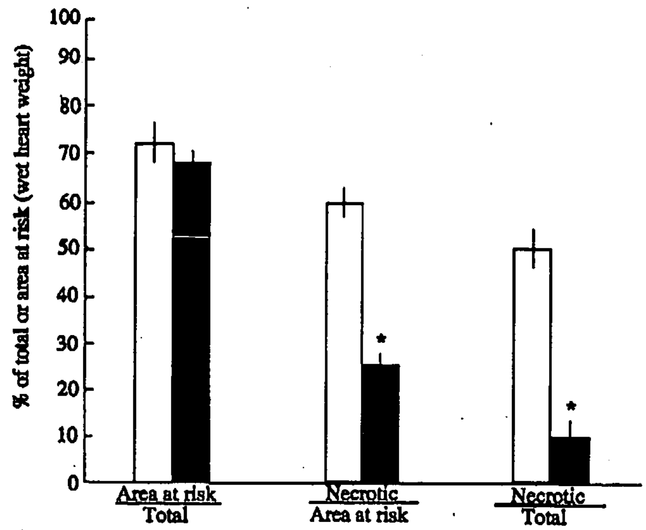

Fig. 2. Effects of anti-tumor necrosis factor-α antibodies (2 mg/kg i.v.) (■) or non-immune serum (0.3 ml i.v. dissolved in a phosphate-buffered saline solution at pH 7.4) (□) on the area-at-risk indexed to total ventricle (area-at-risk/total left ventricle × 100) and necrotic area indexed to area-at-risk (necrotic area/area-at-risk × 100) and to total left ventricle (necrotic area/total left ventricle × 100) as a percentage of wet weight. Bar heights represent mean ± S.D. of six experiments. * $P < 0.001$ vs. MI/R + non-immune serum.

sion was 95% (19 survivors) in the sham MI/R animals given either with the carrier vehicle or antibodies. In contrast, the survival rate was significantly reduced (45%; nine survivors) in MI/R animals treated with the carrier vehicle. Passive immunization with specific antibodies raised against murine tumor necrosis factor-α significantly enhanced the resistance of rats to myocardial ischaemia-reperfusion injury (survival = 80%; 16 survivors).

3.2. Myocardial necrosis

Myocardial necrosis was assessed by staining techniques to determine an anatomical index of the myocardial area jeopardized and that which became necrotic. The area-at-risk, localized by negative staining following perfusions with Evan's blue stain, showed no significant differences between the different MI/R groups, thus indicating that a similar amount of tissue was jeopardized by occlusion of the left coronary artery in each MI/R group (fig. 2). The necrotic area, which was measured by negative staining with nitroblue terazolium, showed that a relatively large amount of myocardial tissue, expressed as a percentage of either the area-at-risk or of the total left ventricle, became necrotic (fig. 2; 61 ± 6 and 52 ± 3%, respectively). Passive immunization with specific antibodies raised against tumor necrosis factor-α reduced myocardial necrosis (necrotic area/area-at-risk 24 ± 3%; necrotic area/total area = 11 ± 4%).

3.3. Serum creatinine kinase activity

Serum creatinine kinase activity was evaluated at the end of reperfusion. In untreated MI/R rats serum creatinine kinase activity was significantly increased when compared to sham MI/R rats (fig. 3). The administration of anti-tumor necrosis factor-α antibodies

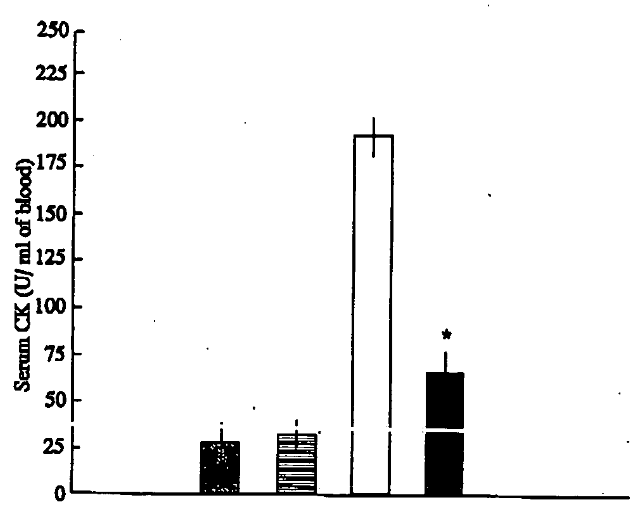

Fig. 3. Serum creatinine kinase activity (CK) at the end of reperfusion in the four groups. Bar heights represent mean ± S.D from six experiments. * $P < 0.001$ vs. MI/R + non-immune serum. First bar: sham MI/R + non-immune serum; second bar: sham MI/R + antibodies; third bar: MI/R + non-immune serum; fourth bar: MI/R + antibodies.

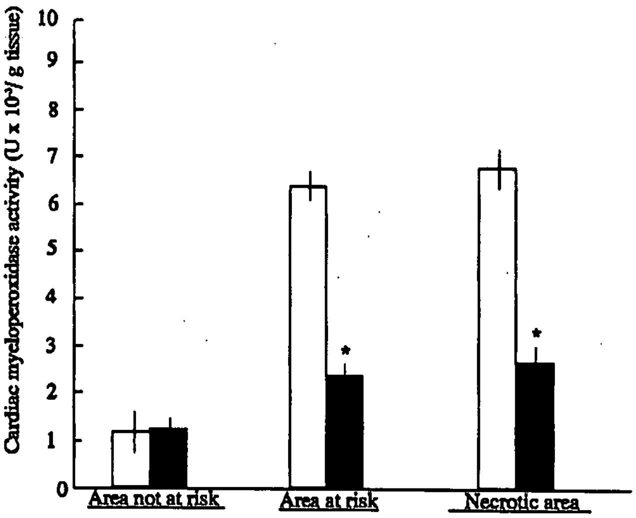

Fig. 4. Effects of anti-tumor necrosis factor-α antibodies (■) or non-immune serum (□) on myeloperoxidase activity in the area-not-at risk, area-at-risk and area of necrosis of rats subjected to myocardial ischaemia-reperfusion (MI/R) injury. Anti-tumor necrosis factor-α antibodies (2 mg/kg i.v.) or non-immune serum (0.3 ml dissolved in a phosphate-buffered saline solution at pH 7.4) were administered 3 h before coronary artery occlusion. Bar heights represent the means ± S.D. from six experiments. * $P < 0.001$ vs. MI/R + vehicle.

resulted in lower serum levels of creatinine kinase, thus confirming that passive immunization exerted cardioprotective effects (fig. 3).

3.4. Myeloperoxidase activity

To quantify the leukocyte response to reperfusion injury, the accumulation of leukocytes was assessed by measuring myeloperoxidase activity (myeloperoxidase) in the three portions of the left ventricle. In the area not-at-risk, myeloperoxidase activity was 1.1 ± 0.5 U $\times 10^{-3}$/g tissue in untreated MI/R rats and 1.2 ± 0.2 U $\times 10^{-3}$/g tissue in MI/R rats pretreated with the specific anti-tumor necrosis factor-α antibodies (fig. 4). In contrast, myeloperoxidase activity was significantly increased in the area-at-risk and in the necrotic area of untreated MI/R rats (6.4 ± 0.6 U $\times 10^{-3}$/g tissue and 6.7 ± 0.8 U $\times 10^{-3}$/g tissue, respectively). Passive immunization with anti-tumor necrosis factor-α antibodies significantly reduced the increase in myocardial myeloperoxidase activity in both the area-at-risk and the necrotic area (2.5 ± 0.3 U $\times 10^{-3}$/g tissue and 2.7 ± 0.5 U $\times 10^{-3}$/g tissue, respectively).

TABLE 1

Tumor necrosis factor-α levels in macrophages of rats subjected t myocardial ischaemia-reperfusion (MI/R) injury.

Each point represents the mean ± S.D. from six experiments. Macrophages were collected after 60 min of reperfusion. N.D. = not detectable.

Treatment	Macrophages tumor necrosis factor-α (U/ml)
Sham MI/R	N.D.
MI/R	145 ± 12

3.5. Macrophage and serum tumor necrosis factor-α

Serum and macrophage tumor necrosis factor-α levels were undetectable in sham MI/R animals and during the occlusion period in MI/R rats treated with

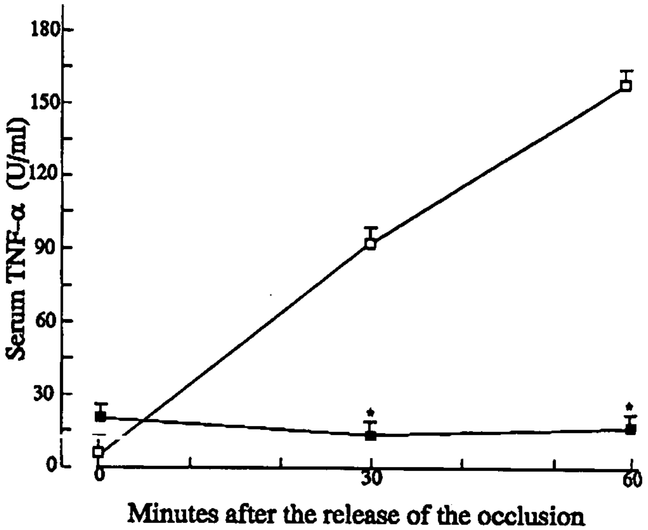

Fig. 5. Time course of tumor necrosis factor-α appearance in the serum of rats subjected to myocardial ischaemia-reperfusion injury (MI/R) and pretreated 3 h before coronary artery occlusion with specific antibodies against tumor necrosis factor-α (2 mg/kg i.v.) (■) or a non-immune serum (0.3 ml i.v. dissolved in a phosphate-buffered saline solution) (□). * $P < 0.001$ vs. non-immune serum.

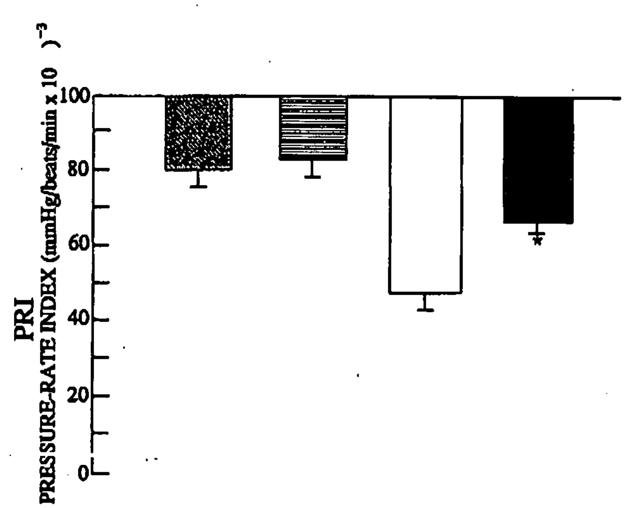

Fig. 6. Pressure rate index in the four experimental groups. The antibodies (2 mg/kg i.v.) or a non-immune serum (0.3 ml/i.v. dissolved in a phosphate-buffered saline solution at pH 7.4) were injected 3 h before the coronary artery occlusion. * $P < 0.001$ vs. MI/R + non-immune serum. First bar: sham MI/R + non-immune serum; second bar: sham MI/R + antibodies; third bar: MI/R + non-immune serum; fourth bar: MI/R + antibodies.

either the non-immune serum or the specific anti-tumor necrosis factor-α antibodies (results not shown). In rats subjected to myocardial occlusion and reperfusion, serum tumor necrosis factor-α progressively increased following the start of reperfusion and reached the maximum increase 60 min after release of the coronary artery (fig. 5). At the end of reperfusion, tumor necrosis factor-α was also significantly increased in the supernatants of peritoneal macrophages collected from MI/R rats (table 1). Passive immunization with a hyperimmune serum containing anti-tumor necrosis factor-α antibodies significantly neutralized tumor necrosis factor-α in the serum (fig. 5).

3.6. *Cardiovascular measurements*

The pressure rate index was comparable for the four groups prior to coronary artery ligation and decreased to a similar extent following thoracotomy (results not shown). At the end of reperfusion the pressure rate index decreased by 20% in sham MI/R rats. The PRI decreased more in untreated MI/R rats than in rats of the sham MI/R groups. However administration of anti-tumor necrosis factor-α antibodies significantly increased the pressure rate index (fig. 6).

4. Discussion

Tumor necrosis factor-α is a cytokine produced by mononuclear phagocytes upon activation by endotoxin or other microbial products (Beutler and Cerami, 1986) and has been implicated in the lethality of Gram-negative septic shock (Tracey et al., 1989; Beutler et al., 1985). This cytokine has pleiotropic activities both 'in vitro' and 'in vivo' and appears to be a common mediator of inflammation and endotoxin shock (Beutler and Cerami, 1987b). Experimental evidence has shown that tumor necrosis factor-α is also involved in the pathogenesis of non-septic low-flow states such as bowel (Squadrito et al., 1992) and hepatic (Colletti et al., 1990) ischaemia-reperfusion injury. The cytokine enhances the adherence of leukocytes to the vascular endothelium, and this latter event is particularly relevant to the development of reperfusion injury. All these findings prompted us to study the role played by tumor necrosis factor-α in myocardial reperfusion injury in the rat. The questions that we raised were: does tumor necrosis factor-α represent an important mediator of myocardial reperfusion injury? Does tumor necrosis factor-α influence leukocyte accumulation in the myocardium during reperfusion 'in vivo', thus confirming the 'in vitro' data? And, finally, are macrophages a major source of tumor necrosis factor-α?

As far as the first question is concerned, our present data indicate that passive immunization with specific antibodies raised against murine tumor necrosis factor-α increases the resistance of rats to the experimental procedures of myocardial ischaemia-reperfusion injury. Survival in the group of rats given the non immune-serum was 45%, whereas rats administered the specific anti-tumor necrosis factor-α showed enhanced resistance to the experimental procedures (survival 80%). Occlusion and reperfusion of the left coronary artery produced marked myocardial necrosis and the passive immunization with specific antibodies directed against murine tumor necrosis factor-α reduced the infarct size. In addition, anti-tumor necrosis factor-α antibodies reduced serum levels of creatinine kinase serum levels, thus confirming the cardioprotective effects of this pretreatment. Finally, the administration of specific anti-tumor necrosis factor-α antibodies reduced the decrease in pressure rate index, in turn suggesting a reduction in myocardial oxygen demand.

During the reperfusion period, the animals subjected to myocardial ischaemia-reperfusion injury had increased serum levels of tumor necrosis factor-α that were neutralized by anti-tumor necrosis factor-α antibody administration. These findings, taken together, strongly support the idea that tumor necrosis factor-α is heavily involved in the pathogenesis of myocardial reperfusion injury.

Leukocyte accumulation plays a crucial role in the development of myocardial damage following reperfusion (Lucchesi, 1990). In our experiment we studied myocardial myeloperoxidase activity to assess leukocyte infiltration, as reported previously (Griswold et al., 1988). Rats subjected to occlusion and reperfusion of the coronary artery showed marked leukocyte infiltration, as indicated by the marked increase in myocardial myeloperoxidase activity. Passive immunization with specific antibodies raised against murine tumor necrosis factor-α decreased the deleterious accumulation of leukocytes in the myocardium. This latter experimental evidence indirectly unmasks the negative role played by the cytokine in myocardial reperfusion injury. More specifically, the cytokine, released by activated cells during the reperfusion period, is responsible for the increased adherence of leukocytes to the ischaemic myocardium. It could be argued that tumor necrosis factor-α, released in the bloodstream, enhances the expression of ICAM-1 and ELAM-1 on the endothelial surface, as reported previously (Bevilacqua et al., 1989), thus promoting leukocyte migration through interendothelial cell junctions and leukocyte accumulation in the subcellular regions. Finally, leukocytes could release deleterious mediators (i.e. free radicals, eicosanoids, platelet-activating factor, etc.) able to induce myocardial cell injury.

Tumor necrosis factor-α is produced in septic shock by macrophages following their activation by endotoxin (Beutler and Cerami, 1987). We therefore investigated

whether tumor necrosis factor-α is produced by macrophages during myocardial ischaemia-reperfusion injury. We found increased tumor necrosis factor-α levels in the peritoneal macrophages collected from rats subjected to myocardial ischaemia-reperfusion injury. These data support the idea that tumor necrosis factor-α is produced by activated macrophages and released during myocardial reperfusion, in turn causing leukocyte infiltration and myocardial injury. However, other cells, such as lymphocytes, endothelial, and smooth muscle cells might also be important sources of tumor necrosis factor-α (Vilcek and Lee, 1991). It has been shown that, following ischaemia caused by clamping of the coronary artery, intracellular Ca^{2+} concentrations increase (Whalen et al., 1974; Shen and Jennings, 1972). During reperfusion, which occurs following clamp release, tissue Ca^{2+} concentrations increase 10 times over control in the first 10 min. Therefore, it could be speculated that an enhanced Ca^{2+} influx may prime macrophages and possibly other cells to synthesize and release the cytokine. Alternatively, it could be hypothesized that stimulation of phospholipase A_2, which follows Ca^{2+} entry during ischaemia, induces eicosanoid synthesis (i.e. thromboxane A_2), which is in turn capable of causing tumor necrosis factor-α production. It has been suggested that calmodulin is involved in cytokine secretion elicited by lypopolysaccharides-stimulated macrophages (Kovacs et al., 1988). Whether this calmodulin-sensitive mechanism, primed by the intracellular calcium overload, also operates during myocardial ischaemia-reperfusion injury requires further investigation.

In conclusion, our results support the idea that tumor necrosis factor-α may be involved in the pathogenesis of myocardial reperfusion injury and suggest that drugs capable of reducing the levels of the cytokine may represent a novel therapeutic approach to the treatment of acute myocardial infarction.

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MECHANISMS OF DISEASE

FRANKLIN H. EPSTEIN, M.D., *Editor*

CACHECTIN: MORE THAN A TUMOR NECROSIS FACTOR

BRUCE BEUTLER AND ANTHONY CERAMI

THE metabolic impact of infectious and neoplastic disease states has long been known to clinicians.[1-4] Invasive diseases may disrupt normal homeostatic mechanisms, both locally and systemically. For example, acute gram-negative infections frequently lead to profound metabolic acidosis and to biphasic changes in plasma glucose concentration, both seen in the context of hypotension, disseminated intravascular coagulation, and widespread tissue injury.[5-12] Chronic infectious diseases, as well as neoplastic diseases, may provoke a severe wasting diathesis, in which negative calorie and nitrogen balance lead to death despite the absence of a large parasite or tumor burden.

It was once widely believed that invasive agents were themselves responsible for these metabolic derangements. In recent years, however, there has been a growing awareness that endogenous mediators are essential elements in the pathogenesis of shock and cachexia alike.

The role of bacterial endotoxin in the pathogenesis of septic shock illustrates this principle. Endotoxin does not exert most of its effects on the host's metabolism directly, nor is it highly toxic to most mammalian tissues. On the contrary, endotoxin elicits the production of a host factor (or factors) that may in turn lead to shock and death. These factors appear to be produced by cells of hematopoietic origin. Transplantation studies in which marrow from endotoxin-sensitive (C3H/HeN) mice was infused into endotoxin-resistant (C3H/HeJ) recipients[13] have shown that sensitivity is conferred by cells of the donor. The macrophage has been suspected to be the cell responsible for endotoxin-induced injury and death, since various facultative intracellular bacteria, capable of eliciting reticuloendothelial hyperplasia, greatly enhance the endotoxin sensitivity of infected animals.[14,15] Moreover, endotoxin-induced macrophages produce, in vitro, a mediator capable of killing endotoxin-resistant mice.[16]

The host's response to endotoxin is perhaps an extreme illustration of its response to many pathogens. Identification of the mediators that confer endotoxin sensitivity would seem to be essential in the design of a specific strategy to arrest the development of shock in sepsis. Moreover, the agent (or agents) conferring endotoxin sensitivity may prove to be a mediator of general inflammation, which is important in the pathogenesis of many human diseases.

THE HISTORY OF CACHECTIN

"Cachectin" is the name applied to a macrophage hormone originally isolated in the course of studies aimed at delineating basic mechanisms of cachexia in chronic disease. In rabbits, trypanosomiasis produces a profound wasting diathesis.[17,18] Infected animals have a low parasite burden, yet become markedly anorectic and lose over half their body weight before dying of infection. Surprisingly, a striking lipemia develops during the final stages of the disease.[17] Rouzer and Cerami noted that the net elevation of plasma lipids was principally attributable to hypertriglyceridemia and that a clearing defect, caused by a systemic reduction in lipoprotein lipase activity, appeared to explain this phenomenon.[17]

Kawakami and Cerami[19] demonstrated that when endotoxin-sensitive (C3H/HeN) mice were given injections of bacterial lipopolysaccharide, systemic suppression of lipoprotein lipase activity and lipemia occurred. Endotoxin-resistant (C3H/HeJ) mice did not exhibit this response. However, lipemia and lipoprotein lipase suppression could be induced in endotoxin-resistant animals when they were injected with the serum of endotoxin-treated C3H/HeN mice. The cellular source of the factor was shown to be the macrophage. Macrophages obtained from sensitive (but not resistant) mice were able to produce a factor suppressing lipoprotein lipase when the cells were stimu-

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lated with lipopolysaccharide in vitro.[20] The factor responsible for this activity was named cachectin in recognition of its suspected role in the pathogenesis of cachexia.

Beutler et al.[21] purified cachectin to homogeneity and found it to be a polypeptide hormone with a subunit size of approximately 17 kilodaltons. Interestingly, cachectin was produced in considerable abundance, accounting for 1 to 2 percent of the total secretory product of endotoxin-activated RAW 264.7 cells,[21] a murine macrophage line. Similar quantities were later shown to be produced by mouse peritoneal macrophages. Very small amounts of the hormone are made by circulating monocytes,[22] unless the latter are "primed" with interferon-gamma, which greatly augments synthesis (unpublished data).

The hormone was shown to bind by means of a high-affinity receptor to adipocytes and myoblasts,[21] as well as a wide variety of other tissues.[23] After injection of endotoxin, cachectin appeared in the circulation within minutes, reached peak levels after two hours, and then rapidly declined in concentration.[23] The half-life of the hormone in the circulation was determined to be approximately six minutes.[23] Initial estimates of the net quantity of cachectin produced by a single animal in response to injection of endotoxin suggested that a rabbit might produce 1 mg of the hormone.[23] Abe and his coworkers[24] found cachectin or "tumor necrosis factor" (TNF) at a serum concentration of 0.3 μM in rabbits in shock. This would suggest that individual animals may actually produce several milligrams of cachectin in response to a lethal dose of lipopolysaccharide. Thus, the quantity of cachectin produced in such a case exceeds the amount required for lethal injury (see below).

The role of cachectin as a mediator of endotoxic shock was suggested by studies in which mice passively immunized against the hormone were found to be protected against the lethal effect of lipopolysaccharide.[25] With the availability of large quantities of recombinant cachectin, essentially free of contaminating endotoxin, it has been possible to examine the direct effects of the hormone in vivo and to determine whether cachectin itself could induce the shock and tissue injury associated with endotoxemia. When infused into rats in quantities similar to those produced endogenously in response to lipopolysaccharide, cachectin causes piloerection, diarrhea, and an ill, unkempt appearance. Typically, hemoconcentration, shock, metabolic acidosis, transient hyperglycemia followed by hypoglycemia, and hyperkalemia are observed. Severe end-organ damage is apparent on both gross examination and light microscopy.[26] The major arteries of the lungs become plugged with thrombi composed primarily of polymorphonuclear leukocytes, and a severe interstitial pneumonitis is present. Acute renal tubular necrosis, as well as ischemic and hemorrhagic lesions of the gastrointestinal tract, follows the administration of relatively low doses of the hormone (100 to 200 μg per kilogram of body weight in rats). In addition, adrenal and pancreatic hemorrhages are commonly noted.[26] Thus, cachectin evokes changes that essentially duplicate the pathologic effects of endotoxin administration.

Early in 1985, Beutler et al. noted that the amino terminal sequence of mouse cachectin was strongly homologous to that reported for human TNF.[27] It was also observed that cachectin and TNF possessed an identical spectrum of bioactivities and were immunologically indistinguishable, suggesting that they were in fact the same molecule.[27] This assumption was soon confirmed by genetic sequence analysis.[28]

The History of TNF

Among the diverse effects induced by endotoxin, few are so striking as the hemorrhagic necrosis of tumors, first noted nearly a century ago by Dr. William Coley, a New York City surgeon who observed the phenomenon in a patient with a sarcoma who contracted an intercurrent streptococcal infection.[29] Coley repeatedly administered bacterial broths, conditioned by the growth of serratia and streptococcus organisms, to patients with cancer in an attempt to induce hemorrhagic necrosis, with mixed results.[30,31] Shear and his colleagues[32-36] studied the phenomenon in animals and isolated the active agent (the "bacterial polysaccharide" — e.g., lipopolysaccharide) from cultures of serratia organisms.

The severe toxicity of lipopolysaccharide precluded its general use as an antineoplastic agent and stimulated a search for modified molecules capable of inducing hemorrhagic necrosis of tumors without causing shock, coagulopathy, and widespread end-organ damage. In 1962, O'Malley et al.[37] reported that serum obtained from mice in shock because of lipopolysaccharide infusion was capable of eliciting hemorrhagic necrosis of a transplantable tumor grown in another animal. A similar observation was made in 1975 in the laboratory of Dr. Lloyd Old.[38] A serum factor, derived from animals primed with bacillus Calmette–Guérin and injected with endotoxin, was found to elicit hemorrhagic necrosis of transplantable tumors in vivo. Intensive studies of this molecule (TNF) were undertaken in the hope that it might present a means of avoiding the toxic effects of lipopolysaccharide while retaining the beneficial (tumorolytic) effect.

TNF was found to be a product of mononuclear phagocytes[39-42] and was observed in several different species.[40,42,43] Interestingly, the factor was found to be cytotoxic to selected tumor-cell lines (including the mouse fibrosarcoma line L-929) in vitro. The L-929 cell cytotoxicity assay allowed purification of TNF. This was achieved by Dr. Bharat Aggarwal and his colleagues,[44] who also succeeded in purifying lymphotoxin, a tumorolytic protein derived from lymphocytes.[45]

The lymphotoxin and TNF genes appear to be products of an ancient tandem duplication event.[46] Both genes are closely linked on human chromosome 6 and, as such, are HLA-linked.[46] In the mouse, the

genes lie within the D region of the H-2 complex, approximately 70 kilobases proximal to the D gene (Fischer-Lindahl K: personal communication). The proteins specified by these genes share approximately 30 percent homology at the amino acid level.[46-48] In addition, they have a highly concordant range of biologic activities and appear to bind to a common recept r.[49] However, lymphotoxin is produced only by T lymphocytes[50] and B lymphoblastoid cell lines[45,51] in response to mitogenic or specific antigenic stimuli. TNF, as noted above, is a product of macrophages, is produced in greatest quantity after exposure to lipopolysaccharide, and is then produced in far greater abundance than lymphotoxin.

Clinical trials of TNF as an antineoplastic agent are currently in progress. No consensus has emerged about the efficacy of the agent. Since TNF (cachectin) is a primary mediator of the lethal effect of endotoxin, it would seem that the therapeutic index of TNF in patients with cancer might differ little from that of lipopolysaccharide.

Thus, two very different lines of investigation — one aimed at isolating an endogenous mediator of tumor necrosis, and another aimed at isolating endogenous mediators of cachexia and shock — led to the realization that both tumor necrosis and endotoxic shock arise through the action of the same macrophage hormone, cachectin/TNF.

The Structure of Cachectin/TNF and the Regulation of Cachectin Expression

The primary structure of cachectin/TNF derived from three mammalian species (rabbits,[52,53] mice,[28,54,55] and humans[47,56]) has now been determined. In each species, cachectin is produced as a prohormone, which appears to be biologically inactive in in vitro assays of lipoprotein lipase suppression and cytotoxicity (unpublished data). The propeptide, which like cachectin itself is extensively conserved, is cleaved at several sites to yield the mature polypeptide.[57] The human propeptide contains 76 additional amino acids at the amino terminal end of the molecule. It is not known at present whether this sequence (or its cleavage products) exhibits a biologic activity responsible for its extensive conservation.

In each species, two cysteine residues are present, apparently connected by a disulfide bridge.[44] This arrangement is lacking in lymphotoxin, which possesses but a single cysteine residue. Nonetheless, it is thought that the two cytokines have retained conformational similarity, since they compete for binding to a common receptor.

Studies of the control of cachectin biosynthesis[58] have suggested that hormone production is tightly regulated. Both transcriptional and post-transcripti nal activation must occur to all w its production.[58] Thus, the chances of inadvertent release of cachectin are minimized.

Glucocorticoid hormones, which strongly antagonize the effects of endotoxin if they are administered before infectious r endotoxic insult,[58] will completely inhibit cachectin biosynthesis, both by diminishing the quantity f cachectin mRNA that is produced in response to lipopolysaccharide and by preventing its translation. Glucocorticoids are effective in preventing cachectin biosynthesis only if they are applied to macrophages in advance of activation. If dexamethasone is added to macrophage cultures after activation by lipopolysaccharide, no inhibiting effect occurs. Thus, the difficulty encountered in mitigating the effects of sepsis by administration of steroids is understandable in molecular terms. Inhibition of cachectin biosynthesis by steroids does not occur once widespread reticuloendothelial induction has been initiated.

The 3′-untranslated region of the cachectin mRNA molecule contains a sequence element composed entirely of A and U (adenosine and uridine) residues, arranged as repeating octameric units [(UUAUUUAU)n].[28] Comparison of human and murine cachectin mRNA reveals that this sequence is conserved in toto over a span of 33 nucleotides, suggesting that it may have a discrete regulatory function. In addition, mRNA molecules encoding other inflammatory mediators, including lymphotoxin, interleukin-1, granulocyte–macrophage colony-stimulating factor (GM-CSF), and most of the interferons (representing all subclasses) also contain UUAUUUAU sequences of varying length in the 3′-untranslated region.[28] Recently, Shaw and Kamen[59] demonstrated that modified beta-globin mRNA molecules, containing the GM-CSF consensus sequence, are markedly destabilized in vivo. The modified globin mRNA is also strongly superinducible; in the presence of cycloheximide, greatly elevated levels of the message are expressed. Thus, it would seem that this sequence is probably involved in control of cachectin-gene expression, acting at a post-transcriptional level.[28,58]

Mechanism of Action

The postreceptor effects of cachectin are known only in general terms. Torti et al.[60] showed that cachectin acts to suppress biosynthesis of several adipocyte-specific mRNA molecules and to prevent morphologic differentiation of pre-adipocytes. Presumably, lipoprotein lipase is one of many enzymes specifically suppressed at a transcriptional level by the action of this hormone. Cachectin also acts to induce the biosynthesis or release (or both) of specific proteins, including Class I major histocompatibility antigen,[61] GM-CSF,[62] and interleukin-1.[63,64] These biochemical effects of cachectin may explain some, but probably not all, of its effects as observed at the level of tissues and organ systems.

The precise mechanism by which cachectin elicits shock and the mechanism by which it induces hemorrhagic necrosis remain unknown. It is likely that the two effects are inextricably related.

As early as 1952, Algire et al.[65] studied the histologic appearance f the necrotizing reaction that occurred within a tumor mass after injection of lipopoly-

saccharide, and concluded that the injury was related to diminished tissue perfusi n.[65] The tumor-necrotizing effect of lipopolysaccharide may be duplicated by mechanical obstruction of regional bl od flow.[65] Moreover, tumors exhibit site-dependent sensitivity to the effects of TNF in vivo. Dermal implants of tumor may be far more liable to destruction than visceral implants (Fidler I: personal communication). Hemorrhagic necrosis appears to arise through vascular events and may be mechanistically unrelated to tumorolysis in vitro.

Recent studies have suggested that cachectin alters the hemostatic properties of the vascular endothelium, inducing the production of a procoagulant activity,[66,67] and inhibiting the expression of thrombomodulin at the cell surface.[66] Both these effects might be expected to favor the accretion of thrombi, leading to disseminated intravascular coagulation at a systemic level and to occlusion of tumor vessels. Among other effects of cachectin on endothelial cells, altered patterns of antigenic expression,[61,68-70] interleukin-1 production,[64,71] and cellular rearrangement[72] in vitro have been observed. It appears that cachectin is directly toxic to vascular endothelial cells.[73] The sum of these effects may explain many of the actions of the hormone in vivo.

It is likely that various vascular beds respond to cachectin in different ways and that certain tumor vessels are more sensitive to the effects of cachectin than the vessels of most normal tissues. It is also quite clear that hemorrhagic necrosis may occur in normal tissues obtained from animals treated with sublethal doses of cachectin.[26] In rats, for example, the cecum is invariably infarcted after administration of cachectin at a dose in excess of 600 μg per kilogram.[26]

Cachectin is most probably a proximal mediator of the effects of lipopolysaccharide, which acts to initiate a large number of events leading to shock and tissue injury. Leukotrienes[74] and platelet-activating factor[75-77] have repeatedly been implicated in the pathogenesis of endotoxic shock, and it is quite possible that cachectin triggers their production. The infiltration of polymorphonuclear leukocytes into numerous organs, particularly the lungs, after cachectin administration may result in part from elaboration of these secondary mediators.

Fluid and electrolyte sequestration invariably accompany (and contribute to) endotoxic shock. Hemoconcentration is observed soon after the administration of cachectin and presumably reflects a rapid decline in intravascular volume. This is most likely attributable to an endothelial lesion, which allows the escape of plasma water and electrolytes to the extravascular space.

The "third space" into which plasma water and electrolytes are ultimately deposited in endotoxic shock is most probably the intracellular compartment. Cachectin has recently been shown to diminish the transmembrane potential f muscle cells.[78] This phenomenon may reflect sodium permeabilization for which the cells cannot compensate or inefficiency of the sodium–potassium-dependent ATPase responsible for maintenance of the electrochemical gradient; either of these conditions would lead to intracellular volume expansion.

In addition to exerting this direct effect, cachectin is known to induce release of interleukin-1 by monocytes and endothelial cells.[63,64] Interleukin-1, in turn, may elicit some of the features that characterize endotoxin poisoning (Dinarello C: personal communication), contributing to the fever, hypotension, neutropenia, and thrombocytopenia that prevail.

The mechanism through which lysis of certain transformed cells occurs in vitro remains an intriguing problem. This phenomenon, once understood in biochemical terms, may conceivably lead to the design of a novel and effective chemotherapeutic strategy.

Cachectin as an Inflammatory Mediator

Cachectin was isolated as a hormone capable of suppressing the expression of lipoprotein lipase and therefore capable of preventing the uptake and storage of exogenous triglyceride. It was found to suppress the expression of several other adipose-specific enzymes,[60] to inhibit the uptake of acetate by fat,[79] and to cause a net loss of triglyceride from fat ("cachexia in vitro").[60] The administration of recombinant cachectin to laboratory animals causes anorexia and weight loss (unpublished data). However, the role of cachectin in the cachexia of chronic diseases remains to be determined, since cachectin cannot be detected in the plasma of cachectic patients. This finding may reflect the relatively low sensitivity of immunoassays that are currently available.

Cachectin (TNF) has also been considered by some as an endogenous antineoplastic agent[80] — e.g., as a potential mechanism of immune surveillance. It is not clear, however, that any tumors can actually induce cachectin biosynthesis, and it is quite clear that most tumors are highly resistant to the hormone's cytotoxic effect.

Irrespective of the roles in which it was originally cast, cachectin has emerged as a mediator of general inflammation, and a variety of recent observations suggest that the molecule may play an important part in diverse human disease processes. Cachectin is an endogenous pyrogen, capable of inducing fever both through a direct effect on hypothalamic neurons and through the peripheral induction of interleukin-1,[63] which in turn elicits fever. Hence, administration of lipopolysaccharide-free preparations of cachectin to rabbits evokes a biphasic febrile response.[63] The initial rise in temperature is attributable to the direct effect of the hormone, whereas the second rise results from interleukin-1 release.

Cachectin also exhibits osteoclast-activating factor activity.[81] In this respect, it again bears a resemblance to interleukin-1, which was earlier shown to possess osteoclast-activating factor activity.[81] And like interleukin-1, cachectin is capable f stimulating synovial-cell production of prostaglandin E_2 and collagenase.[82]

Cachectin activates polymorphonuclear leukocytes, stimulating their adhesion to endothelial-cell surfaces and enhancing their phagocytic activity.[83,84] A separate effect of the hormone on the endothelial cells themselves also promotes neutrophil adhesi n.[83] In vitro these actions undoubtedly reflect the histopathologic changes evoked by cachectin produced in vivo.

Recently, it has also been reported that cachectin (TNF) induces the differentiation of certain myeloid cell lines in vitro.[85,86] In addition, it has been noted that cachectin can induce GM-CSF production by a variety of cell types.[62] However, it has also been noted that cachectin can act to inhibit hematopoiesis directly in in vitro assay systems, decreasing the expression of granulocyte–macrophage colony-forming units,[87,88] erythroid burst-forming units,[88] and granulocyte–erythroid–macrophage–megakaryocyte-forming units.[88] The net effect of the hormone on hematopoiesis in vivo has not been described.

Numerous inflammatory disorders of diverse origins may depend on the production of cachectin, with all its attendant consequences. For example, excessive production of collagenase and prostaglandin E_2 production may lead to the loss of bone and cartilage in rheumatoid arthritis; this entire process may depend in part on the production of cachectin at a local level. Similarly, inflammatory diseases of the central nervous system, gastrointestinal tract, lungs, kidneys, and other tissues may depend on cachectin release. In years to come, a wide variety of inflammatory diseases will undoubtedly be studied in order to determine whether cachectin, produced autonomously or in response to a specific pathogenic stimulus, has an important pathogenic role.

The range of stimuli known to evoke cachectin production is incompletely known at present. Endotoxin remains the most potent stimulus known. However, virus particles[89,90] and certain other biologic agents[91] also trigger cachectin production. With additional study, it seems likely that many infectious agents will be identified as inducers.

It would seem appropriate to ask what beneficial function of cachectin has justified its evolutionary conservation. Recently, it has been shown that sublethal quantities are capable of protecting mice from challenge with an otherwise lethal inoculum of *Plasmodium berghei* (Schofield L: personal communication). It has also been shown that the hormone exerts an antiviral effect in vitro under certain circumstances.[92,93] In addition, C3H/HeJ mice, which cannot produce cachectin[58] because they have a genetic lesion,[94] are far more susceptible to gram-negative infections than normal mice are.[95] Thus, certain infections may be controlled effectively through the action of this and related cytokines.

Relation of Cachectin to Other H rmonal Factors

Through "priming" (e.g., infecting animals with facultative intracellular bacteria such as BCG or *Corynebacterium parvum*), greatly augmented producti n of cachectin[38,40,96,97] may be achieved in vivo after lipopolysaccharide administration. Although this phenomenon remains incompletely understood, it is clear that cachectin production is strongly influenced by other mediators.

Interferon-gamma, which has been shown to activate macrophages, thereby enhancing tumoricidal activity,[98-100] production of hydrogen peroxide,[100,101] phagocytic potential,[100-103] and other defensive functions,[100] also augments the production of cachectin in response to endotoxin.[22,104] Interferon-gamma appears to achieve this effect by enhancing lipopolysaccharide-induced cachectin-gene transcription and cachectin-mRNA translation.[104] It is likely that interferon-gamma (or a related cytokine) contributes to the priming phenomenon.

As previously mentioned, glucocorticoid hormones strongly inhibit cachectin production. The inhibiting effect may be demonstrated in vitro in the presence of cortisol concentrations corresponding to normal human free cortisol levels in vivo. Thus, glucocorticoid hormones may prevent unconstrained release of cachectin, with all its deleterious consequences, which might otherwise occur in the presence of a relatively minor infection. The greatly increased susceptibility to infection of adrenalectomized or otherwise glucocorticoid-deficient patients may reflect the loss of this control mechanism.

Antagonism of Cachectin Action in the Management of Inflammatory Disorders

Therapeutic measures aimed at attenuating the inflammatory response are at present very nonspecific. Glucocorticoid hormones and a variety of cytotoxic drugs can effectively impede inflammation, but also affect host metabolism and arrest the proliferation of many normal host tissues. As a central mediator of inflammation, cachectin is an isolated target for pharmacotherapeutic intervention.

It has been demonstrated that mice treated with a polyclonal antiserum directed against mouse cachectin become resistant to the lethal effect of lipopolysaccharide.[25] Thus, it would seem possible that neutralizing monoclonal antibodies directed against human cachectin may prove to be useful in the treatment of sepsis, particularly in its early stages.

It remains to be seen whether such antibodies (or other cachectin antagonists) will also prove useful in the treatment of other pathologic states in which inflammation has a role. However, it is anticipated that specific neutralization of cachectin and of related cytokines may offer new therapeutic directives with which to treat a broad spectrum of diseases.

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TUMOR NECROSIS FACTOR/CACHECTIN STIMULATES PERITONEAL MACROPHAGES, POLYMORPHONUCLEAR NEUTROPHILS, AND VASCULAR ENDOTHELIAL CELLS TO SYNTHESIZE AND RELEASE PLATELET-ACTIVATING FACTOR

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Platelet-activating factor (PAF)[1] is a mediator of inflammation with a wide range of biological activities (see reference 1 for review). PAF was initially recognized as a product of IgE-sensitized rabbit basophils (2) and was identified with 1-*O*-alkyl-2-*sn*-glyceryl-3-phosphorylcholine (3–5). It was subsequently shown that PAF is synthesized after appropriate stimulation by monocytes/macrophages (6–9), polymorphonuclear neutrophils (7, 10, 11), platelets (12) and endothelial cells (13–15). PAF induces aggregation and degranulation of platelets (2, 16), stimulates contraction of smooth muscle (17), promotes chemotaxis and granule secretion of neutrophils (18–19) and monocytes (20); increases vascular permeability, and alters the vascular tone (21).

It was recently suggested that PAF is a mediator of endotoxic shock (22–24) on the basis of the following observations: (*a*) PAF is produced during endotoxic shock and experimental sepsis by Gram-negative bacteria (23–25); (*b*) infusion of experimental animals with PAF results in hypotension, decrease in cardiac output, and hypovolemic shock (26–28); (*c*) three PAF receptor antagonists (CV3988, kadsurenone, and SRI 63072) inhibit or reverse endotoxin-induced hypotension and, in this way, prolong the survival of rats (22, 23, 29).

Tumor necrosis factor/cachectin (TNF) is a mediator of endotoxic shock (30). Because TNF administration to experimental animals reproduces several aspects of PAF infusion (30), it seems possible that PAF is synthesized in response to

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[1] ***Abbreviations used in this paper:*** acetyl-CoA, acetyl-coenzyme A; APNE, *N*-acetyl-D,L-phenylalanine-β-naphthyl ester; PAF, platelet-activating factor; PBDB, *p*-bromodiphenacylbromide; PC, L-2-phosphatidylch line; PMN, polymorphonuclear neutrophils; TPCK, L-1-tosylamide-2-phenyl thyl chloromethyl ketone.

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TNF. The aim of the present study was to establish whether TNF stimulates synthesis of PAF in cultures of different primary and tum r cell lines. The results obtained show that TNF-treated rat peritoneal macrophages and polymorphonuclear neutrophils (PMN), and human vascular endothelial cells synthesize and release PAF.

Materials and Methods

Cell Cultures. Peritoneal cells were obtained from Lewis rats of 250–300 g according to Bloom et al. (31). The peritoneum was washed with RPMI 1640 medium buffered with 5 mM Pipes containing 0.25% of lipid-free BSA. The cells were washed twice with this medium by 5 min centrifugation at 200 *g*, counted in a hemocytometer, and resuspended in MEM at 10^7 cells/ml. The cells were then plated in 3.5-cm-diam plastic dishes and incubated for 60 min at 37°C. Nonadherent cells were removed by three washes with MEM containing 0.25% BSA; 90–95% of the adherent cells were characterized as macrophages on the basis of positive staining for nonspecific esterase performed on formalin-fixed cells with α-naphtol-AS-D-chloroacetate, specific esterase staining (32), and by phagocytosis of complement-activated zymosan particles (0.2 mg/ml) or of latex beads after incubation for 30 min at 37°C. Rat PMN were cultured as described (7). Endothelial cells were isolated from the human umbilical cord vein, cultured in Iscove's medium supplemented with 15% fetal calf serum (FCS), and characterized as previously described (13). Primary cultures were plated in 3.5-cm-diam plastic dishes, washed, and refed with the same medium every 2 d and 12 h before PAF synthesis assays. These cells were used when confluent after 4–7 d of culture. Human melanoma SK-MEL-109 cells were cultured in F12 and MEM (1:1) with 8% FCS. HeLa cells and human osteosarcoma (HOS) cells were cultured in Dulbecco's medium with 10% FCS. Human foreskin fibroblasts were cultured in F12 medium with 10% FCS and grown to confluency before treatment with TNF.

Synthesis and Release of PAF. For each assay, 2.5×10^5 or 10^6 cells were incubated at 37°C with either human or murine recombinant TNF (hTNF or mTNF), obtained respectively from Dr. Tatsuro Nishihara of the Suntory Institute for Biomedical Research, Osaka, Japan, and from Dr. Walter Fiers, University of Ghent, Ghent, Belgium. In some experiments, macrophages were incubated with the calcium ionophore A23187 and with complement-activated zymosan C (33), or preincubated for 10 min at 37°C with: acetyl-coenzyme A (acetyl-CoA); 2-lyso-PAF (Bachem Feinchemikalien, Bubendorf, Switzerland); EDTA; *p*-bromodiphenacylbromide (PBDB); L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK); and *N*-acetyl-D, L-phenylalanine-β-naphthyl ester (APNE). To label PAF, macrophages were preincubated for 2 h with 15 μCi of [^{3}H]acetyl-CoA (1 Ci/mmol; Amersham Corp., Arlington Heights, IL) or with 2.5 μCi [^{3}H]2-lyso-PAF in 2 ml of MEM containing 0.25% BSA. This preincubation was previously found to yield maximal incorporation of the labeled precursors into PAF (34). After washing, the cells were stimulated with mTNF and the reactions were stopped by adding EDTA to 20 mM. The supernatant was removed and adherent cells were harvested with a rubber policeman in 1 ml of methanol. PAF was extracted from supernatant and cells according to Bligh and Dyer (35), with formic acid added to lower the pH of the aqueous phase to 3.0 (36). In parallel experiments, cell viability was monitored by Trypan blue exclusion and ranged between 89 and 92% after 4 h incubation with different doses of TNF, 95–99% after 1 h incubation with complement-activated zymosan, and 85–90% after 1 h incubation with A23187.

PAF Purification. The supernatants and the methanol fraction obtained from cells were extracted with a 1:1:0.9 (vol/vol/vol) mixture of chloroform, methanol, and water or medium, and the chloroform-rich phase was retained (37). To measure the overall recovery of PAF, 10 nCi of [^{3}H]PAF was added to the cells or supernatants and extracted as described above. The extracted lipids were analyzed by thin layer chromatography (TLC) on silica gel plates 60 F254 (E. Merck, Darmstadt, Federal Republic f Germany) developed with 65:35:6 (vol/vol/vol) chloroform, methan l, and water (37). 1-cm^2 secti ns

of the plates were scraped into glass tubes and the lipids were extracted three times for 30 min with 2 ml of 1:2:0.8 (vol/vol/vol) chl roform, methanol, and water. The silica was removed by centrifugation and the supernatant was adjusted to and extracted with 1:1:0.9 (vol/vol/vol) chloroform, methanol, and water. The chloroform phase was then removed and dried. The dried material was resuspended for quantitation and characterization of PAF in Tris-buffered Tyrode's solution (2.6 mM KCl, 1 mM $MgCl_2$, 1.37 M NaCl, 6 mM $CaCl_2$, 0.1% sucrose, and 1 mM Tris buffer, pH 7.4) containing 0.25% BSA (fraction V; Pentex Biochemicals, Kankakee, IL).

In studies with labeled precursors of PAF, the lipid extracts were chromatographed on 60 F254 TLC plates with 50:25:8.4 (vol/vol/vol/vol) chloroform, methanol, acetic acid, and water (34); 0.5 cm^2 sections were scraped, and radioactivity was counted after addition of scintillation liquid. Commercially available synthetic PAF and 2-lyso-PAF, L-2-phosphatidylcholine (PC) and sphingomyelin from bovine brain, and L-2-lyso-PC from bovine liver (Sigma Chemical Co., St. Louis, MO) were used as standards. The recovery of [^{3}H]PAF after extraction and purification was 96–98%.

PAF Assay. After extraction and purification, PAF was detected by aggregation of washed rabbit platelets prepared as described (38), using an aggregometer. For PAF assay, 5×10^7 platelets were stirred at 37°C in 0.3 ml of Tris-buffered Tyrode's solution supplemented with 0.25% gelatin (Difco Laboratories, Detroit, MI), 0.01 mM indomethacin to inhibit cyclooxygenase, 312.5 mg/liter of creatine phosphate and 152.5 mg/liter of creatine phosphokinase to convert ADP into ATP. In this way, both arachidonic acid– and ADP–dependent platelet aggregation were blocked. PAF concentration was calculated from a calibration curve with 10 μl of various solutions containing synthetic PAF added to rabbit platelets at a final concentration of 1–15 ng/ml. A linear correlation between platelet aggregation and added PAF was observed in a concentration range of 1–10 ng/ml. PAF was quantitated after extraction and purification by TLC (37), and its concentration was calculated on the linear portion of the calibration curve from at least three dilutions of the same sample. This method allowed to quantitate (in nanograms per milliliter) biologically active material of standard PAF.

Characterization of PAF. Biologically active material extracted from cells and supernatant in different experiments was characterized by comparison with PAF obtained from sensitized rabbit basophils (37) and with synthetic PAF by the following criteria (39): (*a*) induction of platelet aggregation by a pathway independent from both ADP- and arachidonic acid/thromboxane A_2–mediated pathways; (*b*) specificity of platelet aggregation inferred from the inhibitory effect of 5 μM SRI63072 (29) and CV3988 (40), two different PAF receptor antagonists; (*c*) physicochemical characteristics such as inactivation by strong bases and phospholipase A_2, but resistance to phospholipase A_1 (41), acids, weak bases, and 5 min heating in boiling water. The methods used were previously described in detail (39).

Results

The experiments shown in Fig. 1 established that rat peritoneal macrophages adherent to plastic dishes release PAF in response to TNF. The macrophages were incubated for 1 h with 20 ng/ml of mTNF and PAF was assayed after purification from cells and medium as described in Materials and Methods. The macrophages produced PAF in amounts comparable to those obtained during phagocytosis of complement-activated zymosan C, and ~75% of maximal synthesis obtained with the calcium ionophore A23187 (Fig. 1). The level of cell-associated PAF was approximately equal to that in the medium, suggesting that TNF was a potent inducer of both PAF synthesis and release. The material extracted from cells and medium had biologic and physicochemical characteristics identical to those of synthetic PAF and of PAF released from IgE-sensitized rabbit basophils. It induced platelet aggregation in an ADP- and arachidonic

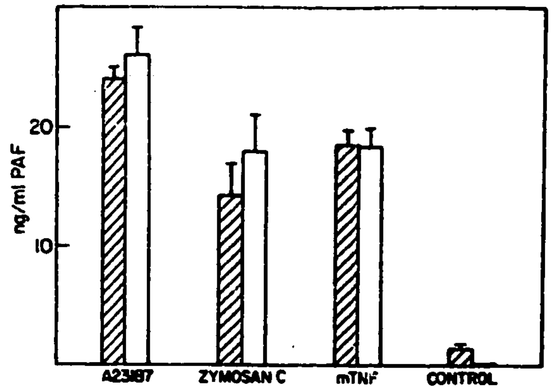

FIGURE 1. Synthesis and release of PAF by rat perit neal macrophages treated f r 1 h with 1 µg/ml of A23187, 0.2 mg/ml f zymosan C, 20 ng/ml f mTNF or untreated. 10^6 cells were used for each experiment. Cell-associated PAF, shaded columns; supernatant PAF, blank columns. In this and the f ll wing figures, PAF concentration is referred to 1 ml of cell culture medium and to the corresponding cell aliquot to allow comparison of the amount of PAF released with that remaining cell associated. Vertical bars indicate the standard deviation of the mean of different experiments ($n = 3$). The assay of PAF was carried out in duplicate for each experiment.

acid-independent way, which was specifically inhibited by the PAF receptor antagonists SRI63072 and CV3988 (29, 40). PAF activity was destroyed after base-catalyzed methanolysis (0–1% residual activity) or after treatment with phospholipase A_2 (0–3% residual activity), indicating the presence of an ester linkage at *sn*-2 (13, 37, 41–43). Treatment with phospholipase A_1 did not inhibit PAF activity, suggesting the presence of an ether bond at *sn*-1 (13, 41). The PAF activity was resistant to treatment with acids or weak bases (21, 37). After base-catalyzed methanolysis or digestion with phospholipase A_2, treatment with acetic anhydride restored 88.7 ± 7% ($n = 3$) of the biologic activity. The PAF obtained from macrophages had the same R_f (0.21) in TLC as synthetic PAF, using as solvent chloroform/methanol/water (65:35:6). No PAF activity was detected in any other TLC fraction. The R_f of PAF changed to 0.10 by using in TLC a solvent with different polarity (chloroform/methanol/water; 65:35:4), indicating that PAF extracted from macrophages is a polar lipid.

Evidence that PAF was synthesized de novo by mTNF-treated rat peritoneal macrophages was obtained by following the incorporation of radioactive precursors. TLC analysis of the lipid fraction extracted 1 h after addition of mTNF from macrophages preincubated with [^{3}H]acetyl-CoA showed one main peak of radioactivity that comigrated with synthetic PAF; this peak was absent from the lipid fraction of untreated macrophages (Fig. 2*A*). Another experiment showed that exogenous 2-lyso-PAF could be a substrate for PAF synthesis. TLC analysis of the lipid fraction of macrophages preincubated with [^{3}H]2-lyso-PAF plus 0.1 mM unlabelled acetyl-CoA and then treated with mTNF showed three peaks of radioactivity (Fig. 2*B*). The first peak comigrated with 2-lyso-PAF, the second peak with PAF, and the third peak with PC. In the lipid fraction of untreated cells the PAF peak was absent, indicating that [^{3}H]2-lyso-PAF was only converted into other phospholipids comigrating with PC. It should be pointed out that, in these experiments, efficient conversion of 2-lyso-PAF into PAF required the addition of acetyl-CoA, since only a small peak of radioactivity comigrated with PAF when the lipid fraction of cells preincubated with [^{3}H]2-lyso-PAF alone and treated with mTNF was analyzed by TLC (data not shown).

Time Course and Dose-Response of PAF Synthesis. The following experiments measured the time course of PAF synthesis and release int the supernatant by macrophages treated with 20 ng/ml of mTNF (Fig. 3). After 30 min PAF was mainly cell associated, whereas after 1 h it was present in equal amounts in cells

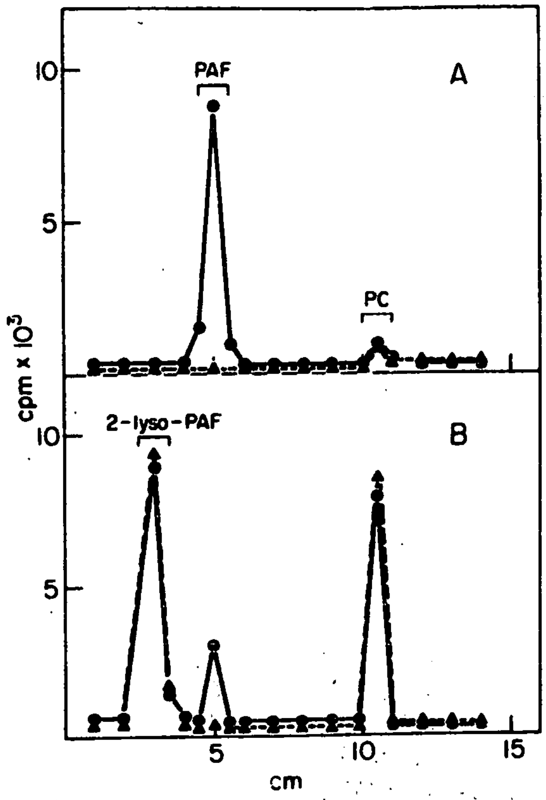

FIGURE 2. Representative TLC analysis of PAF synthesized by rat peritoneal macrophages stimulated f r 1 h with 20 ng/ml of mTNF after 2 h preincubation with 15 μCi/ml of [^{3}H]acetyl-CoA (*A*) or with 0.1 mM unlabelled acetyl-CoA and 2 μCi/ml [^{3}H]2-lyso-PAF (*B*). The lipids were extracted from cells and supernatant (35) and analyzed by TLC using as solvent chloroform/methanol/acetic acid/water (50:25:8:4). The plates were divided into 30 sections of 0.5 cm each and counted as described in Materials and Methods. The pattern obtained with TNF-stimulated cells (●) and control unstimulated cells (▲) is shown. In parallel experiments, labeled PAF, PC, and 2-lyso-PAF were chromatographed as standards; the position of these compounds is indicated. Three experiments were performed with similar results on cell-associated and supernatant lipids.

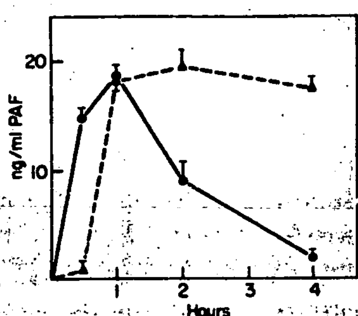

FIGURE 3. Time course of PAF synthesis and release by rat peritoneal macrophages stimulated with 20 ng/ml of mTNF. Cell-associated PAF (●) and PAF released in the supernatant (▲) are shown; 10^6 cells were used for each time point.

and supernatant. Cell-associated PAF decreased afterwards, whereas that in the supernatant increased sharply after 1 h but declined slightly after 4 h (Fig. 3). This indicated that maximal synthesis of PAF occurred within 1 h of TNF addition, and that PAF was gradually released into the supernatant from 1 to 4 h. Some PAF was also inactivated during this time, since its total amount (cell associated plus supernatant) decreased. The synthesis of PAF and its release by macrophages in response to different doses of mTNF or hTNF was also measured (Fig. 4). PAF was extracted from cells and supernatant 1 h after TNF addition. The amount of cell-associated PAF was approximately equal to that found in the supernatant at all TNF concentrations tested. Significant PAF synthesis was detected with 1 ng/ml of mTNF and nearly maximal synthesis with 10 ng/ml. Fivefold greater concentrations of hTNF were required to obtain a similar response.

The Effect of Precursors and Enzyme Inhibitors on PAF Synthesis. The effect of precursors of PAF and of inhibitors of cyclooxygenase or phospholipase A_2 was

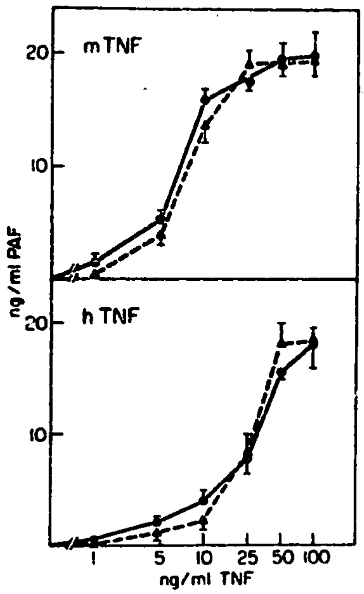

FIGURE 4. Dose response of PAF synthesis and release by rat peritoneal macrophages stimulated for 1 h with mTNF (*A*) or hTNF (*B*). The cell-associated PAF (●) and that released into the supernatant (▲) by 10^6 cells treated with different concentrations of TNF are shown.

TABLE I
Effect of PAF Precursors and Enzyme Inhibitors on the Synthesis of PAF by Rat Peritoneal Macrophages

Compound added	Concentration	PAF	
		Cell associated	Released
	μM	$\mu g/ml$	
Acetyl-CoA	0	15.5 ± 3.3	15.8 ± 3.7
	1	15.2 ± 3.3	16.2 ± 3.3
	10	20.5 ± 3.5	22.2 ± 1.8
	50	23.1 ± 2.5	23.7 ± 2.6
	100	24.4 ± 2.4	26.4 ± 3.3
2-Lyso-PAF	1	15.8 ± 2.9	16.8 ± 3.3
2-Lyso-PAF + acetyl-CoA	1 + 100	27.8 ± 1.7	28.5 ± 3.4
APNE	100	5.3 ± 2.5	2.4 ± 0.6
TPCK	10	0.9 ± 0.9	<0.1
EDTA	100	0.7 ± 0.6	<0.1
Indomethacin	10	16.3 ± 1.5	15.3 ± 1.4
PBDB	1	4.7 ± 1.5	3.5 ± 1.4

For each experiment, 10^6 rat peritoneal macrophages were preincubated for 10 min with the compounds indicated and then incubated for 1 h with 20 ng/ml of mTNF. Cell-associated PAF and that released into the culture medium were measured as described in Materials and Methods. Cell viability was between 85 and 92% in experiments with different inhibitors. The mean ± SD of different experiments (n = 3) is indicated.

investigated in the experiments shown in Table I. Acetyl-CoA stimulated in a dose-dependent manner the synthesis and release of PAF by peritoneal macrophages treated for 1 h with 20 ng/ml of mTNF. It should be pointed out that no PAF activity was recovered from the supernatant of untreated macrophages in control experiments (n = 8) carried out in the absence of acetyl-CoA. In contrast, minimal amounts of PAF (1.2 ± 0.8 ng/ml) were extracted from macrophages treated with 0 1 mM acetyl-CoA. When these cells were preincu-

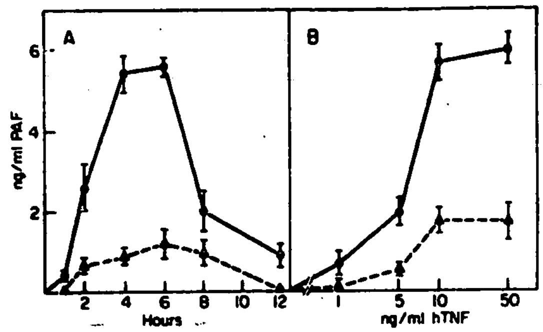

FIGURE 5. Time course (*A*) and d se response (*B*) of PAF synthesis and release by human endothelial cells stimulated with hTNF. The cell-associated PAF (●) and that released in the supernatant (▲) after treatment with 10 ng/ml of hTNF or after 4 h incubation with different concentrations f hTNF is shown; 2.5 × 10^5 cells were used for each time point and hTNF concentration. The vertical bars indicate the standard deviation of the mean of different experiments ($n = 5$).

bated with 2-lyso-PAF before mTNF addition, no significant increase in PAF production was observed (Table I). However, addition of both 2-lyso-PAF and 0.1 mM acetyl-CoA increased significantly the synthesis and release of PAF. In the absence of mTNF, these compounds increased the level of cell-associated PAF to 2.2 ± 0.6 ng/ml, but did not cause PAF release into the medium (data not shown).

Two inhibitors of serine proteases that block the cytocidal activity of TNF in human and murine cells (44) reduced both synthesis and release of PAF (Table I). The effect of the reversible inhibitor APNE, which is a substrate of proteases (44), was less pronounced than that of TPCK, an alkylating agent that is an irreversible inhibitor of proteases (45). TPCK is, on a molar basis, the most effective antagonist of the cytocidal activity of TNF (44). This inhibitor abolished the release of PAF into the medium. A similar effect was obtained with the cation chelator EDTA (Table I) and with ethylene-*bis*-(oxyethylenenitrilo)-tetracetic acid, a chelator with high specificity for Ca^{2+} (data not shown), suggesting that synthesis and release of PAF may require Ca^{2+} entry into TNF-treated cells. The inhibitor of cyclooxygenase indomethacin had no effect on PAF synthesis (Table I). In contrast, the inhibitor of phospholipase A_2 PBDB drastically reduced synthesis and release of PAF. These findings suggest that PAF is produced upon TNF-mediated activation of phospholipase A_2.

Production of PAF by Human Vascular Endothelial Cells and Rat PMN. Cultures of human endothelial cells obtained from the umbilical vein were treated for different times and with different concentrations of hTNF. Significant PAF synthesis was observed after 2 h of treatment, and reached its maximum after 4 h, declining sharply afterwards (Fig. 5*A*). Most of the PAF synthesized was cell associated, since only ~20% was found in the supernatant fraction. The dose-response of PAF synthesis showed nearly maximal synthesis, with 10 ng/ml of hTNF and significant synthesis with 1–5 ng/ml (Fig. 5*B*). Only ~20% of the PAF synthesized was found in the supernatant fraction at all concentrations of hTNF tested. We also assayed synthesis and release of PAF in rat PMN treated for 1 h with 20 ng/ml of mTNF: 5.7 ± 1.7 ng of PAF were found associated with 10^6 PMN, and 3 ± 1.1 ng were released per milliliter of medium. In contrast, no detectable amounts of PAF were recovered after 1–4 h of treatment with 10 ng/ml of hTNF from human foreskin fibroblasts and tumor cells such as SK-MEL-109 melanoma, HOS osteosarcoma, and HeLa cells, or from their culture media (data not shown). It appears from these results that synthesis and release of PAF are a specialized cell response to TNF. The reasons for the

different efficiency of PAF release between macrophages and end thelial cells are presently unknown.

Discussion

TNF was initially detected in the serum of endotoxin-treated animals as the mediator of the necrosis of some transplantable tumors in mice (46). It was subsequently reported that TNF is cytostatic or cytotoxic for certain tumor cells (47). However, several lines of evidence indicate that this is not the only biologic activity of TNF. This factor can stimulate fibroblast proliferation (48, 49), is pyrogenic (50), affects lipid metabolism (51), and modulates several functions of endothelial cells (52, 53). Furthermore, recent reports indicate that TNF is a mediator of inflammatory reactions. TNF is chemotactic for monocytes and PMN (54), stimulates phagocytosis (55), adherence to endothelium (52), and superoxide production by these cells (56, 57), and induces procoagulant activity in cultured human vascular endothelial cells (58). Furthermore, data from experimental animals suggest that TNF production plays a primary role in endotoxin shock (59).

A variety of cells that play a role in inflammation have been shown to produce PAF. This factor may be synthesized and released, after appropriate stimulation, from cells of the monocyte/macrophage series (6–9), PMN (7, 10, 11), platelets (12), and endothelial cells (13–15). Therefore, PAF may be involved in a variety of immunopathological reactions triggered by different cellular effectors. In addition to its role in acute inflammatory reactions, PAF may be a mediator of shock when it is released intravascularly in massive amounts within a short period of time (23–27, 37). The present study shows that, after stimulation with TNF, rat peritoneal macrophages, human endothelial cells, and rat PMN synthesize and release PAF.

Appropriate stimuli are necessary to synthesize and release PAF (1, 60). The calcium ionophore A23187 (6, 33) and the phagocytosis of zymosan (61, 62) or of immunocomplexes (9) triggers PAF production from monocytes/macrophages. The present study shows that the amount of PAF synthesized by TNF-treated rat peritoneal macrophages is comparable to that produced in response to phagocytic stimuli. The response to TNF is rapid, since already at 30 min considerable amounts of PAF are detected in these cells (Fig. 3). The subsequent decrease of cell-associated PAF in macrophages continuously incubated with mTNF indicates that its synthesis is a transient response. This can be either a consequence of downregulation of TNF receptors or of other regulatory mechanisms that limit the synthesis of PAF.

Previous studies on a variety of inflammatory cells (8, 13, 63–65), including zymosan-stimulated macrophages (33), show that PAF is synthesized by two enzymatic steps: (*a*) hydrolysis of 2-lyso-PAF by phospholipase A_2; and (*b*) acetylation of 2-lyso-PAF at position 2 by an acetyl transferase. The first step requires Ca^{2+} and is inhibited by PBDB (8). The second step is detected by the incorporation of acetate into PAF by cells incubated with labelled acetyl-CoA (8, 13, 33, 66–68). The biosynthesis of PAF by TNF-stimulated macrophages involves these two metabolic steps (Fig. 2). The requirement for Ca^{2+}, suggested by the effect of EDTA, and the inhibitory effect of PBDB (Table I) indicates

that phospholipase A_2 is involved in 2-lyso-PAF hydrolysis from membrane lipids. Addition of this compound to TNF-stimulated macrophages does not result in increased PAF synthesis, whereas acetyl-CoA stimulates PAF synthesis. These results are in agreement with previous findings (33), which suggest that the limiting step for PAF biosynthesis is the concentration of activated acetate available as acetyl-CoA.

The reduced synthesis of PAF observed in the presence of the protease inhibitors APNE and TPCK suggests that some proteolytic activity is required for TNF stimulation of PAF synthesis. These compounds protect human and murine cells incubated with the inhibitor of protein synthesis cycloheximide from the cytocidal activity of TNF (44). It seems therefore possible that this activity and the synthesis of PAF are elicited through some common step in response to the signalling of TNF-receptor complexes. Phospholipase A_2 activity is thought to be regulated in vivo by potent inhibitory membrane-associated proteins designated calpactins or lipocortins (69). Synthesis of PAF may possibly require some proteolytic cleavage of these proteins to activate phospholipase A_2.

Identical amounts of PAF are synthesized by rat peritoneal macrophages in response to optimal concentrations of either mTNF or hTNF (Fig. 4). However, mTNF stimulates these macrophages at lower concentrations than hTNF. This finding may be explained by the species-specificity of TNF. By measuring binding of radiolabelled mTNF and hTNF to homologous and heterologous receptors of murine and human cells, Smith et al. (70) have shown that mTNF binds with almost equal affinity to both cell types, whereas hTNF binds with higher affinity to human cells than to murine cells. This difference in binding to receptors is reflected by a correspondingly lower cytostatic activity of hTNF on murine L cells than on human HeLa cells (70). In the present experiments carried out on rat cells, mTNF appears to be more effective than hTNF. Therefore, it seems likely that TNF receptors on rat cells bind mTNF with greater affinity than hTNF. This concept is supported by the finding that maximal PAF synthesis by human endothelial cells is obtained with hTNF concentrations similar to those that induce maximal response in rat peritoneal macrophages with mTNF, namely, 10 ng/ml (Figs. 4*A* and 5*B*). This TNF concentration (0.6 nM) results in occupancy of a large fraction of cellular receptors, based on a K_d at 4°C of 2×10^{-10} for the hTNF and mTNF receptors (70).

The synthesis of PAF in response to TNF appears to be a specialized response of cells involved in inflammation. Large amounts of PAF are synthesized by macrophages and endothelial cells, but macrophages appear to release PAF rapidly, whereas 80% of the PAF synthesized remains associated with endothelial cells (Fig. 5). The time course of PAF synthesis is also slower for endothelial cells than for macrophages. However, the endothelial cells synthesize PAF for a few hours, in contrast to macrophages, which show a transient synthesis. The observation that in a continuous incubation with TNF endothelial cells respond differently from macrophages supports the hypothesis that PAF synthesis is regulated by specific mechanisms rather than by downregulation of TNF receptors due to receptor-mediated endocytosis. These findings have to be confirmed with other macrophage and endothelial cell systems, but they suggest that regulatory networks control the duration of PAF synthesis and release in response

to TNF. The possibility that PAF itself could promote TNF synthesis was tested in rat peritoneal macrophages. Treatment of these cells with 5–50 ng/ml of PAF for 1–4 h did not result in appreciable release of mTNF into the culture medium (our unpublished observati ns). This finding suggests that TNF and PAF production are not interrelated.

The presence of TNF in the serum of patients with meningococcal meningitis and/or septicemia has recently been reported (71). TNF was detected in 10 of 11 patients who died, but in only 8 of 68 survivors (71). Clinical signs of severe meningococcal disease are leukopenia, thrombocytopenia, and hypotension. These symptoms may be caused by release of PAF mediated by the TNF in serum. It seems likely that PAF release may play a significant role in both local and systemic inflammatory responses to TNF production. Further studies are necessary to establish the relevance of TNF-mediated PAF synthesis in different immunopathologic conditions.

Summary

Murine tumor necrosis factor (mTNF) stimulates production of platelet-activating factor (PAF) by cultured rat peritoneal macrophages in amounts comparable to those formed during treatment with the calcium ionophore A23187 or phagocytosis of zymosan. The cell-associated PAF that was released into the medium was identical to synthetic PAF, as determined with physicochemical, chromatographic, and enzymatic assays. Furthermore, de novo synthesis of PAF by macrophages was demonstrated by the incorporation of radioactive precursors such as [^{3}H]acetyl–coenzyme A or [^{3}H]2-lyso-PAF. Macrophages incubated with mTNF for 4 h synthesized PAF only during the first h of treatment. At this time, the amount of cell-associated PAF was approximately equal to that released into the medium. The cell-associated PAF decreased afterwards, whereas that in the medium did not correspondingly increase, suggesting that some PAF was being degraded. The response of rat macrophages to different doses of mTNF and human TNF (hTNF) was examined. Maximal synthesis of PAF was obtained with 10 ng/ml of mTNF and 50 ng/ml of hTNF. This finding may be explained by a lower affinity of hTNF for TNF receptors of rat cells. The hTNF stimulated production of PAF by human vascular endothelial cells cultured from the umbilical cord vein. The time course of PAF synthesis was slower than that observed with macrophages, with maximal production between 4 and 6 h of treatment. Optimal synthesis of PAF was obtained with 10 ng/ml of hTNF. Only 20–30% of the PAF synthesized by endothelial cells was released into the medium, even after several hours of incubation. Synthesis of PAF in response to TNF was also detected in rat polymorphonuclear neutrophils, but not in human tumor cells and dermal fibroblasts. Therefore, production of PAF is a specialized response that is transient in macrophages continuously treated with TNF, and that appears to be controlled by unidentified regulatory mechanisms.

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Cytokines in Acute Myocardial Infarction: Selective Increase in Circulating Tumor Necrosis Factor, Its Soluble Receptor, and Interleukin-1 Receptor Antagonist

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Summary: Cytokines play a pathogenetic role in a variety of infective and inflammatory diseases. In the present study, we had two objectives: (a) to define the kinetics of tumor necrosis factor (TNF) in plasma after acute myocardial infarction (AMI) in patients treated with early thrombolysis, and (b) to measure other cytokines, interleukin-1 (IL-1) and TNF receptor antagonists, in plasma. TNF-α, but not IL-1β or IL-8, was present in plasma of 6 of 7 patients with severe AMI (Killip class 3 or 4). No TNF (<50 pg/ml) was detected in a group of 11 patients with uncomplicated myocardial infarction (Killip class 1) or in control patients without AMI. Soluble TNF receptor type I and IL-1 receptor antagonist (IL-1Ra) were also significantly increased in the group with severe AMI compared with those with uncomplicated AMI. Circulating TNF is increased only in AMI complicated by heart failure at hospital admission. This finding may have diagnostic and therapeutic relevance. **Key Words:** Myocardial infarction—Cytokines—Tumor necrosis factor—Interleukin-1.

Circulating levels of tumor necrosis factor (TNF) have been measured in patients after acute myocardial infarction (AMI) (1,2). Recently, Levine and colleague (3) reported high concentrations of TNF in cachectic patients with chronic heart failure, secondary to coronary artery disease in 19 of 33 of them. Cytokines play a pathogenetic role in a growing number of infective and inflammatory diseases. In particular, TNF has been shown to be a key mediator in the pathogenesis of septic shock, as indicated by the protective effect of anti-TNF antibodies in various animal models (4,5). Recently, TNF was also reported to be induced during hepatic ischemia/reperfusion in rats (6). In this experiment model, inhibition of TNF with antibodies not only protected against liver ischemic damage, but also prevented pulmonary damage secondary to liver ischemia/reperfusion, indicating that released TNF might play a role in the complications of the disease. Experimental data suggest that cytokines can mediate myocardial ischemia/reperfusion damage (7,8). In general, these data suggest that TNF might be induced not only by infective or inflammatory stimuli. The reported induction of TNF and interleukin-1 (IL-1) production by human monocytes exposed to hypoxia might be the basis of production of TNF in ischemia/reperfusion (9). Another important issue is whether other cytokines might be increased, in addition to TNF.

Much attention is being paid to endogenous inhibitors. These include IL-1 receptor antagonist (IL-1Ra), a protein that has high homology with IL-1 but no IL-1 activity and acts as a classic receptor antagonist (10), and the soluble form of the

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TNF receptor (sTNFRtI) (11), which inhibits the activity of TNF by competing with the membrane-bound receptor. Both inhibitors have been shown to be protective in animal models of septic shock (12,13) and are being tested in clinical trials. Both IL-1Ra and sTNFRtI are increased after treatment with endotoxin, indicating their potential role as regulators of cytokine action (14,15). We measured the levels of circulating TNF and other cytokines (IL-1 and IL-8) as well as of sTNFRtI and IL-1Ra in patients admitted to the coronary care unit (CCU) early after onset of symptoms of AMI and related them with severity of cardiac failure at entry.

MATERIALS AND METHODS

All patients (n = 23) were consecutively admitted to the CCU or cardiology divisions of three regional hospitals (Caserta, Magenta, and Udine). We studied 7 patients early after AMI complicated by severe congestive heart failure (severe AMI), identified as being in Killip classes 3 or 4 at entry. Patients received systemic thrombolysis with recombinant tissue plasminogen activator (r-tPA) or streptokinase (SK) ≤3 h 21 min after onset of symptoms. Exclusion criteria for the present study were onset of symptoms >10 h before examination, steroid therapy, chronic and acute diseases (tumors, rheumatoid arthritis, systemic lupus erythematosus, acute inflammatory diseases). Patient characteristics are shown in Table 1. Blood was sampled at entry and at 6, 12, 24, 36, 48, and 72 h after admission to the CCU. Blood was collected on EDTA and immediately spun, and the plasma was frozen at −20°C until analyzed.

A group of 11 patients who had uncomplicated AMI at entry (Killip class 1) was studied with the same exclusion criteria (uncomplicated AMI). Patients with uncomplicated AMI also received systemic thrombolysis with r-tPA or SK ≤3 h 3 min after onset of symptoms (Table 1). Blood was sampled at entry, 6 h after admission to the CCU, and at hospital discharge, and was processed as already described.

Another group of 6 patients without AMI (controls) was referred to the cardiology clinic for diagnostic examinations, no signs of acute coronary syndrome, cardiac failure, or inflammatory disease (five supraventricular arrhythmias and one stable angina). A single blood sample was obtained and was handled as all other samples.

TABLE 1. *Patients' characteristics*

Patient/sex/body weight (kg)/Age (yr)	Time from onset of symptoms	Outcome at discharge	Killip class at entry	Peak CK	Time of peak CK (h, min)	Site of infarct	Thrombolytic	ASA	Heparin
Severe AMI									
GP/M/70/68	2, 40	Dead	4	1,699	8, 40	Inferior	r-tPA	No	No
MA/F/75/76	6, 00	Alive	3	641	6, 00	Inferoposterior	r-tPA	Yes	Yes
AP/F/56/81	5, 00	Alive	4	2,077	14, 00	Non-Q	r-tPA	Yes	Yes
CP/M/82/59	3, 30	Alive	3	722	12, 30	Anterior	SK	Yes	Yes
GR/M/106/53	3, 20	Dead	4	942	21, 30	Anterior	r-tPA	No	Yes
ML/F/72/71	2, 00	Dead	4	150	—	Inferoposterior	r-tPA	Yes	No
CP/M/75/47	1, 00	Dead	4	57	1, 00	Non-Q	r-tPA	No	No
Mean 77/65	3, 21			898	10, 36				
SD 15/12	1, 43			752	7, 5				
Uncomplicated AMI									
CM/M/75/69	2, 55	Alive	1	33	—	Anterior	NO	Yes	No
CL/M/?/50	3, 25	Dead	1	1,497	12	Anterior	SK	Yes	Yes
D'AF/M/76/59	1, 00	Alive	1	1,269	6	Inferior	r-tPA	Yes	No
GA/M/67/69	9, 00	Alive	1	2,872	6	Anterior	SK	Yes	Yes
SC/F/50/71	1, 00	Alive	1	482	9	Anterior	SK	No	Yes
TC/M/78/87	2, 30	Alive	1	1,250	—	Inferior	r-tPA	Yes	No
BA/M/73/79	4, 00	Dead	1	98	—	Anterior	SK	Yes	No
CG/M/77/84	1, 30	Alive	1	435	12	Anterior	SK	Yes	No
MM/M/95/79	2, 00	Alive	1	939	18	Inferior	SK	Yes	No
AR/M/86/53	1, 00	Alive	1	574	—	Inferior	r-tPA	Yes	No
CC/M/90/60	5, 00	Alive	1	1,800	18	Inferior	SK	Yes	No
Mean 77/69	3, 03			1,023	12				
SD 13/12	2, 23			839	5				

No AMI	Diagnosis
CR/F/70/63	Paroxysmal atrial flutter
VR/M/60/62	Supraventricular tachycardia
BU/M/81/62	Stable angina
RP/M/91/62	Paroxysmal atrial fibrillation
MV/M/65/66	Paroxysmal atrial fibrillation in hypertensive disease
RA/F/70/61	Chronic atrial fibrillation in congenital heart disease
Mean 73/63	
SD 11/2	

AMI, acute myocardial infarction; ASA, acetylsalicylic acid; CK, creatine kinase; r-tPA, recombinant tissue plasminogenen activator; SK, streptokinase.

IL-1β was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Amersham: sensitivity of the assay in our experimental conditions 5 pg/ml). IL-8 was measured by an ELISA kit from British Bio Technology (sensitivity of the assay in our experimental conditions 20 pg/ml). TNF-α was measured by an ELISA kit using a capture murine monoclonal antibody (a gift from D. Wallach, Weizmann Institute, Rehovot, Israel) and a rabbit policlonal antibody (a gift from P. Ghiara, Sclavo, Siena, Italy) with a sensitivity of 50 pg/ml. To confirm that the TNF detected by ELISA was bioactive, TNF was also measured in some samples (in this case, in serum samples) with a bioassay using L929 cells as a target (16). IL-1Ra and sTNFRtI were measured by competitive-binding radioimmunoassay using iodinated recombinant IL-1Ra or sTNFRtI and a rabbit polyclonal antibody to IL-1Ra or sTNFRtI (17,18) (sensitivity in the present assays 125 pg/ml). Five patients from the severe AMI group, 5 from the uncomplicated AMI group, and 5 controls (no AMI) were also studied for IL-1Ra and sTNFRtI. All assays were run in duplicate, and results are expressed as picograms per milliliter. All data are mean ± SD. Statistical comparisons between groups were made with Kruskal-Wallis nonparametric test followed by Sachs test for multiple comparisons ("Easy Stat" program, running on Macintosh SE/30).

RESULTS

Patients' characteristics

In the severe AMI group, two of seven infarctions were anterior (Table 1); peak creatine kinase (CK) serum concentrations were 898 ± 752 U/ml. In the uncomplicated AMI group, six of 11 infarctions were anterior (Table 1); peak CK concentrations were 1,023 ± 839 U/ml. Four patients in the severe AMI group died in the CCU of unresponsive cardiac failure; 2 in the group with uncomplicated AMI died of cardiac rupture (documented by autopsy). The other 3 patients in the severe AMI group were discharged alive with major functional impairment owing to cardiac failure (New York Heart Association class 3).

Cytokine levels

Plasma levels of IL-1β and IL-8 remained undetectable in all groups, including the 7 patients with severe AMI. On the contrary, a marked increase in plasma TNF-α was observed in 6 of 7 patients in the severe AMI group (Fig. 1). Peak TNF levels (≤3,600 pg/ml in 1 patient) were detected within 12 h of AMI and returned to undetectable levels by 24 h. Levels of TNF remained undetectable in both the uncomplicated AMI group and the control group (Table 2). The 3 survivors of the severe AMI group, examined 6 weeks after AMI, had no measurable concentrations of TNF despite the severity of their disease. Mean peak concentrations and ranges are shown in Table 2.

To investigate whether the TNF-α detected by ELISA was biologically active, serum samples obtained from 3 severe AMI patients at admission were assayed for TNF by ELISA and by biologic assay (cytotoxicity on L929 cells). In this case, serum was used because plasma obtained in the presence of anticoagulants is not suitable for the bioassay. All three samples were positive for both immunoreactive TNF and bioassay (data not shown).

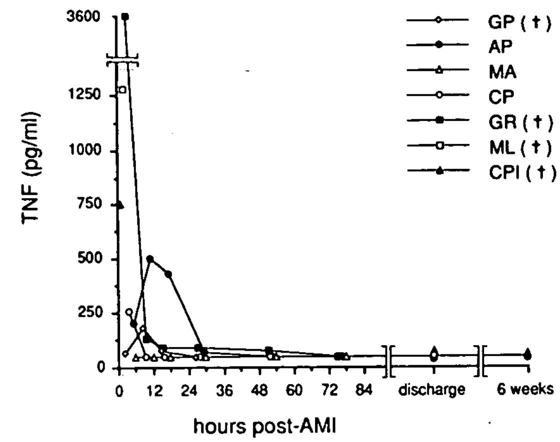

FIG. 1. Plasma tumor necrosis factor (TNF) concentrations in 7 patients early after acute myocardial infarction (AMI) complicated by severe congestive heart failure at entry (severe AMI group). Limit of detection of the TNF enzyme-linked immunosorbent assay used was 50 pg/ml († = dead).

Levels of cytokine inhibitors

IL-1Ra was markedly increased in patients with severe AMI (Fig. 2A) as compared with those with uncomplicated AMI (Fig. 2B). IL-1Ra in severe or uncomplicated AMI peaked at ~12 h and decreased sharply in the next 12 h. Low but detectable levels of IL-1Ra were noted in AMI group (Table 2).

The kinetics of sTNFRtI levels in the two AMI groups are shown in Fig. 3. High sTNFRtI levels were noted in the severe AMI group as compared

TABLE 2. *Peak plasma TNF, IL-1Ra, and sTNFRtI concentrations (pg/ml) in severe AMI, uncomplicated AMI, and control groups*

Cytokine	Severe AMI	Uncomplicated AMI	No AMI
TNF	1,094 ± 1,177[a]	<50	<50
	180–3,600[b]	—	—
	n = 7	n = 11	n = 5
IL-1Ra	4,766 ± 2,953[c]	2,026 ± 1,271[c]	219 ± 89
	1,580–8,500	570–4,100	115–345
	n = 5	n = 5	n = 5
sTNFRtI	1,184 ± 404[c]	162 ± 160	62 ± 139
	740–1,640	<125–364	<125–310
	n = 5	n = 5	n = 5

TNF, tumor necrosis factor; IL-1Ra, interleukin-1 receptor antagonist; sTNFRtI, soluble form of TNF receptor.

[a] Mean ± SD.

[b] Range.

[c] $p < 0.01$ versus No AMI group. Statistical analysis was not applied to TNF since it was undetectable in two of three groups.

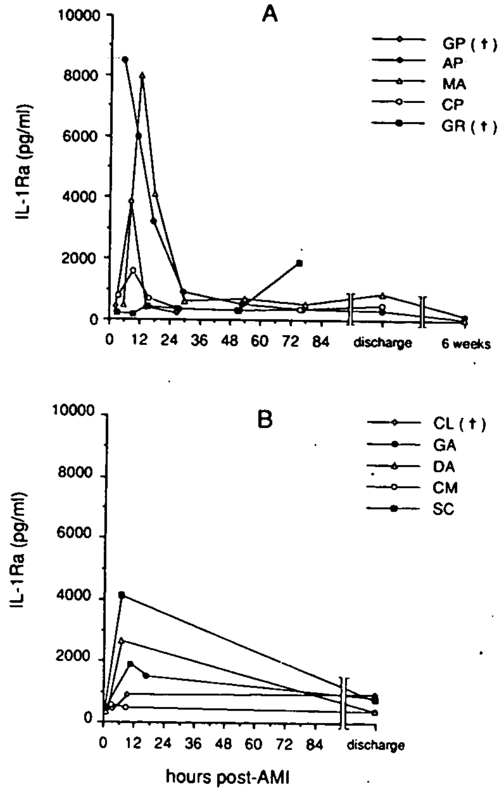

FIG. 2. Plasma interleukin-1 receptor antagonist (IL-1Ra) concentrations in 5 patients early after acute myocardial infarction (AMI) complicated by severe congestive heart failure at entry (severe AMI group) **(A)** and 5 uncomplicated AMI patients **(B)**. Limit of detection for the IL-1Ra radioimmunoassay was 125 pg/ml († = dead).

with patients with uncomplicated AMI, and the time course was more variable than that of TNF (Fig. 1) and IL-1Ra (Fig. 2). Indeed, some patients in the group with severe AMI had a long-lasting increase in sTNFRtI level (Fig. 3A). In the control group, sTNFRtI levels were undetectable (<125 pg/ml) in 4 of 5 patients (Table 2).

DISCUSSION

Our data prove that TNF is present at high levels only in patients with AMI complicated by severe impairment of left ventricular function at entry; no TNF is detected in those without severe impairment. The circulating TNF levels were present for no more than 24 h after onset of symptoms. As compared with results of a previous study (2), TNF peaked much earlier (<12 h vs. 48–84 h after onset of pain), possibly because all our patients had been thrombolyzed early after onset of symptoms, according to the recommended therapeutic guidelines for AMI.

Our data probably reflect a different phenomenon from that described by Levine and colleagues (3). They reported high circulating levels of TNF in the bioassay at only one time point in the life of patients with cardiac cachexia.

Peak TNF levels observed in severe AMI patients are higher than those observed in human volunteers injected with a pyrogenic dose of endotoxin (19). On the other hand, IL-8 induced at levels comparable to those of TNF in human volunteers injected with endotoxin was not detected in AMI patients. We may have missed the peak for this cytokine and, even for patients with the shortest time between symptoms and admission, the levels may already have returned to basal at the first time point. This is unlikely, however, because published data on kinetics indicate that IL-8 is induced, at least by endotoxin, with a kinetics similar to that of TNF and at almost identical levels (19). Therefore, the increase in plasma TNF in AMI patients probably reflects a stimulation acting with a mechanism different from that of endotoxin. Hypoxia can act as a TNF inducer (9), but other biochemical pathways can be important in production of TNF, including increase in intracellular cyclic GMP (20), platelet-activating factor (21), lactic acidosis (22), and α-adrenoceptor stimulation (23). TNF can be produced by a variety of cells, including monocytes-

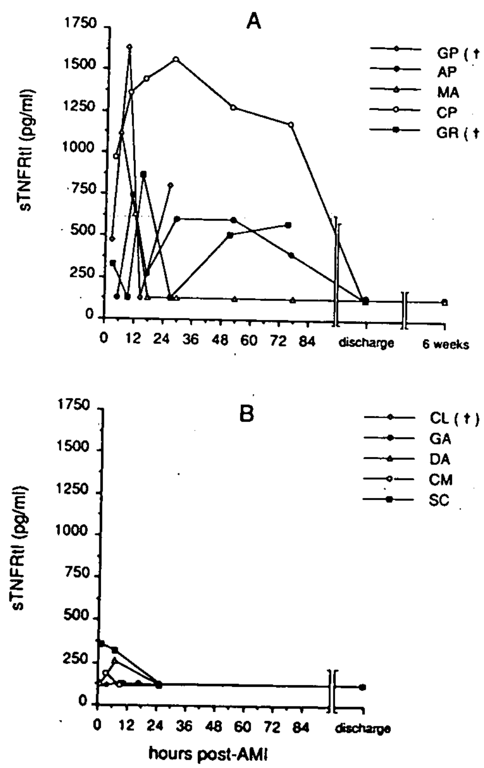

FIG. 3. Plasma soluble form of tumor necrosis factor receptor (sTNFRtI) concentrations in 5 patients early after acute myocardial infarction (AMI) complicated by severe congestive heart failure at entry (severe AMI group) **(A)** and 5 uncomplicated AMI patients **(B)**. Limit of detection of the sTNFRtI RIA radioimmunoassay was 125 pg/ml († = dead).

macrophages, endothelial cells, mastocytes, and many others, and the mechanism of induction might be different for the different cell types (4). However, because all the processes described might be activated in all types of AMI, independent of severe left ventricular dysfunction, the difference we observed in TNF production cannot be easily explained, although high circulating TNF may reflect the intensity of shock in the severe AMI group.

The induction of IL-1Ra and sTNFRtI we report is in agreement with data from a growing body of literature indicating that cytokine inhibitors are induced under the same pathologic conditions in which cytokines themselves are induced. One obvious implication for this finding is that, when one constructs hypotheses regarding the possible pathogenetic role of a cytokine, one should also measure the levels of the respective inhibitor. This suggests at least two possible sites for pharmacologic intervention, i.e., inhibition of TNF production or stimulation of TNF-receptor shedding.

In consideration of the proinflammatory actions of TNF, its production might be important in defining the pathogenesis of AMI-associated inflammation (9). Indeed, a case of AMI in a cancer patient treated with TNF was reported, possibly indicating a direct pathogenetic role of TNF in AMI (24). This hypothesis is supported by the observation that protection by TGF-β from AMI damage in rats is mediated by its inhibitory action on TNF production during ischemia (10). More recently, pretreatment with TNF was shown to protect rats against myocardial ischemia/reperfusion injury (25) (assessed by increase lactate dehydrogenase release from perfused hearts), probably by induction of manganous superoxide dismutase. This is consistent with the hypothesis that TNF might be a mediator of myocardial ischemia/reperfusion injury since TNF induced desensitization to a second challenge with a lethal dose of TNF (26). Further studies will be required to seek (a) a relation between TNF and other biohumoral indexes of AMI extension and/or severity, (b) effects of pharmacologic interventions on TNF production, (c) effects of reperfusion/thrombolysis, and (d) the prognostic meaning of circulating TNF levels. In this regard, we should remember that antagonists or inhibitors of TNF are available for clinical studies in patients with septic shock syndrome. Furthermore, it is important to consider the possibility that TNF production or its activity might be modulated with drugs recommended after AMI. In particular, nonsteroidal anti-inflammatory agents have been shown to potentiate TNF production by inhibiting prostaglandin-mediated negative feedback (19). Heparin infusion in human volunteers was reported to induce the release in circulation of a soluble form of the TNF receptor (27) and soluble TNF receptors are effective inhibitors of TNF of therapeutic interest, as shown by their protective effect in animal models of TNF-mediated toxicity (13).

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Eosinophil Cationic Granule Proteins Impair Thrombomodulin Function

A Potential Mechanism for Thromboembolism in Hypereosinophilic Heart Disease

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Abstract

Thromboembolism is a prominent but poorly understood feature of eosinophilic, or Loeffler's, endocarditis. Eosinophil (EO) specific granule proteins, in particular major basic protein (MBP), accumulate on endocardial surfaces in the course of this disease. We hypothesized that these unusually cationic proteins promote thrombosis by binding to the anionic endothelial protein thrombomodulin (TM) and impairing its anticoagulant activities. We find that MBP potently (IC_{50} of 1–2 μM) inhibits the capacity of endothelial cell surface TM to generate the natural anticoagulant activated protein C (APC). MBP also inhibits APC generation by purified soluble rabbit TM with an IC_{50} of 100 nM without altering its apparent K_d for thrombin or K_m for protein C. This inhibition is reversed by polyanions such as chondroitin sulfate E and heparin. A TM polypeptide fragment comprising the extracellular domain that includes its naturally occurring anionic glycosaminoglycan (GAG) moiety (TMD-105) is strongly inhibited by MBP, whereas its counterpart lacking the GAG moiety (TMD-75) is not. MBP also curtails the capacity of TMD-105 but not TMD-75 to prolong the thrombin clotting time. Thus, EO cationic proteins potently inhibit anticoagulant activities of the glycosylated form of TM, thereby suggesting a potential mechanism for thromboembolism in hypereosinophilic heart disease. (*J. Clin. Invest.* 1993. 91:1721–1730.) Key words: Eosinophils • thrombomodulin • glycosaminoglycan • cationic protein • major basic protein • eosinophil peroxidase • eosinophil cationic protein

Introduction

Peripheral blood eosinophilia, irrespective of its cause, is frequently complicated by a morbid and potentially lethal form of endocarditis characterized by eosinophils (EOs)[1] adhering to and infiltrating the endocardium, mural thrombosis, endocardial damage, and embolism (1, 2). Such eosinophilic endocarditis may lead rapidly to death from thromboembolic complications, evolve over months or years to cause progressive endocardial and myocardial damage culminating in congestive heart failure, or resolve over years leaving residual endomyocardial fibrosis (1–3). This unusual form of endocarditis, although rare in temperate climates, causes 10–20% of all cardiac deaths in tropical Africa and Southeast Asia, where chronic hypereosinophilia, caused by endemic parasitic infestations, is common (4).

Mechanisms underlying the pronounced thromboembolic diathesis that characterizes both the acute and chronic phases of eosinophilic endocarditis are poorly understood. However, EO granule proteins have been implicated in the pathogenesis of this disorder. EO-specific granules are comprised almost entirely of four unusually cationic (pI > 11) proteins (major basic protein [MBP], eosinophil peroxidase [EPO], eosinophil cationic protein [ECP], and eosinophil-derived neurotoxin [EDN]) that function as potent but nonspecific cytotoxins (reviewed in reference 5). Patients with eosinophilic endocarditis have degranulated circulating EOs (6) as well as high (up to micromolar) serum levels of MBP (7). Moreover, endomyocardial biopsies taken at various stages of this disease uniformly demonstrate dense endocardial and small vessel endothelial surface deposition of MBP (8), ECP (8), and EPO (9).

Endothelial (10) and endocardial (10) cells actively participate in maintaining an anticoagulant surface, in part through their expression of the 105-kD transmembrane protein thrombomodulin (TM) (10–12). Endothelial cell surface TM exerts an anticoagulant effect by avidly (K_d 0.5 nM, [11, 12]) binding circulating thrombin to (*a*) curb its fibrinogen-cleaving activity, (*b*) potentiate the interaction of antithrombin III with thrombin, and (*c*) alter the substrate specificity of thrombin, accelerating greatly its proteolytic activation of circulating protein C to activated protein C (APC). APC, in turn, is a powerful anticoagulant serine protease that, in conjunction with protein S, terminates the procoagulant activity of Factors Va and VIIIa (10–12). Of note, the complete TM molecule is quite anionic (pI $\approx$ 4 [13]), in part because of extensive posttranslational glycation of the large extracellular domain of TM with an unusual hypersulfated, chondroitin sulfate E–like moiety (14–20). This bulky polyanionic domain strongly influences all three known anticoagulant functions of TM (14–23).

We hypothesized that eosinophilic cationic granule proteins deposited on endocardial and endothelial surfaces bind electrostatically to the anionic extracellular domain of TM, impair TM anticoagulant function, and thereby contribute to the prominent thromboembolic diathesis that typifies eosinophilic endocarditis. To test this hypothesis, we determined the effect of purified human EO granule proteins upon the ability of endothelial cell-bound TM, isolated full-length TM, and the extracellular domain of TM to generate APC and impair the fibrinogen-cleaving activity of thrombin.

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1. *Abbreviations used in this paper:* APC, activated protein C; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EO, eosinophil; EPO, eosinophil peroxidase; GAG, glycosaminoglycan; Gla-domainless protein C, γ-carboxyglutamic acid–domainless protein C; MBP, major basic protein; TM, thrombomodulin.

J. Clin. Invest.

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Volume 91, April 1993, 1721–1730

Methods

Materials

Chromogenic substrates S-2366 and S-2388 were obtained from Kabi Vitrum (Franklin, OH). Benzamidine-free bovine protein C and antithrombin III were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Bovine plasma thrombin (2,500 NIH units/mg protein), cycloheximide, porcine rib cartilage chondroitin sulfate A, and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO). Hanks' buffered salt solution and Dulbecco's modified DMEM medium were obtained from Gibco BRL Life Technologies Inc. (Grand Island, NY). Squid cartilage chondroitin sulfate E, super special grade, was obtained from Seikagaku America, Inc. (Rockville, MD). Purified, detergent-solubilized rabbit thrombomodulin and Gla (γ-carboxyglutamic acid)-domainless protein C were generously provided by N. L. and C. T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). The eosinophil granule basic proteins MBP, EPO, ECP, and EDN were purified to physical homogeneity from granule preparations derived from EOs of patients with hypereosinophilic syndrome as previously described (24–26). Human thrombomodulin TMD-105 and TMD-75 were kindly provided by John Parkinson (Eli Lilly and Co., Indianapolis, IN). Human umbilical vein endothelial, porcine aortic endothelial, and human aortic endothelial cells were obtained from collagenase-treated blood vessels as previously described (27) and maintained in DMEM supplemented with penicillin, streptomycin, L-glutamine, and 15% heat-inactivated FCS (Gibco, Grand Island, NY). Cells were grown to confluence and used for experiments ~ 1 wk after initial seeding.

Methods

Immunofluorescent localization of major basic protein in cardiac sections. Formaldehyde-fixed and parafin-embedded tissue microtome sections were obtained at autopsy from a 70-yr-old male with clinical eosinophilic endocarditis and hypereosinophilia related to the presence of a pulmonary carcinoma that secreted a potent eosinophilopoietic factor, as we have previously described (28). Sections were stained for the presence of MBP using a polyclonal rabbit anti-MBP antibody and visualized by indirect immunofluorescence using a goat anti–rabbit IG antibody conjugated with FITC as previously described (29). As a control, serial sections were stained with protein A affinity-purified normal rabbit IgG and showed no fluorescence.

Assay of APC generation by endothelial monolayers. Tissue culture medium was aspirated from endothelial monolayers, which were then washed three times with H/H buffer (Hanks' buffered salt solution supplemented with 1 mM magnesium and calcium and 20 mM Hepes buffer, pH 7.4). Monolayers were overlaid with 200 μl of either H/H buffer or H/H containing increasing concentrations of MBP. Preliminary experiments established that the inhibitory effect of MBP on APC generation by endothelial monolayers was already maximal by 10 min. To ensure that the MBP/TM interaction was complete, however, monolayers were exposed to MBP for 30 min. Supernatant buffer was then aspirated and the monolayers subsequently washed two times in 1 ml of 37°C H/H and overlaid with 500 μl of H/H supplemented with 3 mM $CaCl_2$, 500 nM protein C, and 1.5 nM thrombin. Plates were then incubated at 37°C for 90 min, whereupon APC generation was terminated by the addition of 20 μl of 6 μM antithrombin III, vortexed and incubated 5 min further. This 90-min incubation, based on the work of others utilizing endothelial cell monolayers (23, 30, 31), is necessary to obtain optimal sensitivity. APC generation is linear over the 90-min incubation period (not shown). The resulting mixture was then briefly centrifuged to remove cellular debris and transferred to a cuvette containing 0.400 ml of 0.1 molar NaCl, 0.02 molar Tris, pH 7.4, and 0.1% BSA and 50 μl of either S-2238 or S-2366 (both at 400 nM final). The initial APC generation was then assayed spectrophotometrically by conversion f the chromogenic substrate at 405 nM in a 37°C spectrophotometer cell based on the initial rate of optical density change. Negative controls consisted of empty plastic wells treated in a parallel fashion. Values obtained from these controls was then subtracted from the rates measured for wells containing m nolayers.

Endothelial cell monolayer regeneration of APC-generating capacity after washout of MBP with intact or impaired protein synthetic capacity. 1-cm^2 monolayers (48-well plate) of porcine aortic endothelial cells were aspirated free f tissue culture medium and washed three times with warmed H/H as described above. Wells were then overlaid with 100 μl of H/H with or without 10 μg/ml cycloheximide, then further supplemented with either buffer or 3.3–5 μM MBP and incubated a further 30 min. At this point, monolayers were either washed free of unbound MBP and buffer and assayed for APC-generating capacity using S-2366 as described above or, alternatively, overlaid with 500 μl Iscove's DMEM plus 15% FCS with or without the continued presence of cycloheximide, as appropriate. At various time points thereafter (1–16 h), these latter monolayers were then washed free of supernatant medium and assayed for APC-generating capacity. By 16 h, both in the presence and absence of MBP, the cycloheximide-treated monolayers were visibly altered and had low viability as judged by trypan blue exclusion criteria, so no data is shown from this time point.

Effect of intracellular hypokalemia upon MBP-mediated inhibition of APC generation by endothelial monolayers. 1-cm^2 confluent monolayers of porcine aortic endothelial cells were left normokalemic or rendered intracellularly hypokalemic by treatment with nigericin and exposure to hypokalemic buffers as described by Larkin et al. (32). Tissue culture medium was aspirated from monolayers, which were washed either with buffer B (15 mM Hepes, 100 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgSO_4$) or buffer B supplemented with 4 mM KCl. Monolayers were then overlaid with 500 μl of normokalemic buffer r with hypokalemic buffer containing 4 μM nigericin and incubated 45 min at 37°C before being washed either with normo- or hypokalemic buffer. Monolayers were then assayed for their capacity to generate APC in the presence of 2 nM thrombin, 500 μM protein C, and 3 mM $CaCl_2$ in potassium-free H/H buffer for 3 h at 37°C. After quenching of APC generation with antithrombin III, APC was quantitated using the chromogenic substrate S-2366 as previously described.

Inhibition of rabbit TM APC generation by MBP and EPO. To 50 μl H/H were added 10 μl 30 mM $CaCl_2$ and 10 μl 20 nM rabbit TM and either 10 μl of H/H buffer or H/H buffer containing various concentrations of MBP or EPO. The solution was vortexed and allowed to incubate at room temperature for 10 min, whereupon 20 μl of 2.5 μM bovine protein C and 10 μl of 20 nM bovine thrombin were added, vortexed, and incubated 10 min at 37°C before addition of 10 μl of 60 μM AT-III. After 5 min more of incubation at 37°C, the entire mixture (110 μl) was transferred to a cuvette and 290 μl of cuvette buffer containing 400 nM S-2366, mixed, and assayed for initial rate of APC generation spectrophotometrically at 405 nM.

Effect of MBP on thrombin dependence of protein C activation by soluble rabbit-TM. 10 μl of 10 μM rabbit TM, 10 μl of 15 mM $CaCl_2$, and 10 μl of 375 nM MBP were added to wells on a 96-well microtiter plate (previously treated with 0.1% Tween 20 detergent and rinsed to render surfaces hydrophilic), mixed, and allowed to incubate 10 min at room temperature. The plate was then allowed to warm to 37°C and 10 μl of 2.5 μM protein C was added and mixed. 10 μl of thrombin at various concentrations was then added, the wells were mixed again, and the plate was incubated 10 min at 37°C. During these 10 min of incubation the rate of APC generation was constant under these conditions (not shown). Therefore, APC generated at 10 min represents accurately an initial rate of APC generation that was used to calculate kinetic parameters, as has been done previously (18, 22). 10 μl of 30 μM AT-III was then added, mixed, and incubated 5 min further at 37°C. APC generation was quantitated by adding 150 μl of cuvette buffer containing S-2366 at a final concentration of 400 μM and quantitated by initial rate as assayed at 405 nm on a Thermomax microtiter V_{max} plate reader (Molecular Devices Corp., Menlo Park, CA). Another set of wells was composed as described above with the exception that buffer was substituted for the rabbit TM; values obtained from these wells were subtracted from those obtained from the wells with

rabbit TM at each thrombin concentration to correct for TM-independent cleavage f S-2366 by thrombin.

Determination of apparent K_m of thrombin/rabbit TM complex for protein C. 10 μl of 450 nM MBP or H/H buffer was combined with 10 μl of 12 nM rabbit TM and incubated for 10 min at room temperature, whereupon 30 μl of various concentrations of protein C and 10 μl of 0.6 nM thrombin were added in a 96-well microtiter plate, mixed, and incubated 10 minutes at 37°C. Final conditions were 3 mM $CaCl_2$, 75 nM MBP, and 2 nM rabbit TM. APC generation was terminated by addition of AT-III and assayed using S-2366 as above.

Kinetics of MBP interaction with TM-dependent APC generation. Data from Fig. 5 were analyzed with nonlinear regression analysis (Statistics, Version 5.2, SYSTAT Inc., Evanston, IL) to avoid the hazards of linear analysis related to error distribution (33). Data shown are plus or minus standard deviation.

Polyanion reversal of MBP impairment of rabbit TM. 10 μl of 10 nM rabbit TM was combined with 10 μl of 1 μM MBP in a 96-well plate microtiter well and incubated 10 min at room temperature, subsequent to which 10 μl of various concentrations of chondroitin sulfate A, chondroitin sulfate E, or heparin or H/H buffer was mixed in and the resulting mixture incubated 45 min further at 37°C. 10 μl of 2.5 μM bovine protein C and 10 μl of 10 nM bovine thrombin were then added, mixed, and incubated 10 min before addition of AT-III and assay of APC generation as described above. The final calcium concentration was 3 mM.

Effect of MBP on APC generation by human TM fragments, TMD-105, and TMD-75. 20 μl of 2.5 μM bovine protein C, 40 μl of H/H buffer, and 10 μl of 20 nM TMD-75 or TMD-105 were mixed with 10 μl of either buffer or the indicated concentration of MBP and 10 μl of 50 mM $CaCl_2$, then incubated 5 min at room temperature. The specimen was warmed to 37°C in a water bath and 10 μl of 20 nM bovine thrombin was added to initiate generation of APC. After 10 min, the reaction was terminated with AT-III, incubated 5 min, and assayed for APC using the chromogenic substrate S-2366 in a final volume of 500 μl in a 1-cm cuvette. The values obtained from TM-free controls were subtracted from each data point. Final conditions were 500 nM bovine protein C, 2 nM bovine thrombin, 2 nM thrombomodulin, and 5 mM calcium.

MBP interactions with the capacity of TM to prolong the thrombin clotting time. All reagents were made up in H/H plus 0.15% Lubrol PX supplemented with 1.25 mM $CaCl_2$. 100 μl H/H was mixed with 60 μl of 100 nM TM (either rabbit TM, TMD-105, or TMD-75) and 30 μl of MBP at 10 times its final concentration (0.5 or 1.0 μM), mixed, and incubated 10 min at 37°C. 30 μl of 50 nM bovine thrombin was then added and the mixture incubated 10 min further at 37°C subsequent to mixing with 60 μl of 10 mg/ml of fibrinogen and initiating the thrombin clotting time. Formation of clot was determined in a fibrometer (Becton Dickinson and Co., Cockeysville, MD). Each determination was made at least in triplicate.

Results

As shown in Fig. 1, endocardial surfaces can accumulate dense deposits of eosinophil cationic granule proteins even at the earliest stage of eosinophilic endocarditis, before morphologically evident damage to endocardium occurs. Fig. 1 *A* shows a hematoxylin-and-eosin–stained section of tissue specimen obtained from the right atrial wall of a 70-yr-old male who developed high grade hypereosinophilia and eosinophilic endocarditis caused by a pulmonary carcinoma that secreted a potent eosinophilopoietic factor, as we have previously described (28). The right atrial endocardial wall appears normal although the lumen is in part obliterated by a cellular clot (labeled *C*), which is composed almost entirely f EOs and has apparently detached from the endocardium (labeled *E*) during the fixation process. Fig. 1 *B* shows a serial tissue secti n stained for the presence f MBP by indirect immunofluorescence. Note the bright endocardial MBP deposits, the staining intensity of which rivals that f intact EO granules in the adjacent cellular clot.

To model the endocardial deposition of ECPs seen in eosinophilic endocarditis, we exposed intact endothelial monolayers from three sources to MBP or a buffer control for 30 min, washed away unbound MBP, then assayed their capacity to support generation of APC in the presence of thrombin and protein C. After exposure to MBP, immunofluorescent staining of MBP-treated, but not buffer-treated, monolayers with anti-MBP antibody demonstrated bright cell surface and matrix MBP localization (not shown). As shown in Fig. 2, MBP, the predominant constituent of EO-specific granules (50% of total protein [34]), potently inhibits TM-dependent APC generation by porcine aortic, human umbilical vein, and human aortic endothelial monolayers. The 50% inhibitory concentration (IC_{50}) is 0.5–2 μM, within the range of MBP found circulating in the serum individuals with high grade eosinophilia (35). At concentrations > 5 μM MBP there is no discernible APC generation. This complete abrogation by MBP of the ability of endothelial cells to support thrombin-dependent APC generation is not attributable to a direct cytotoxic effect of MBP on endothelial cells, interference with the assay for APC, or proteolysis of cell surface TM by proteases contaminating our MBP preparation because in experiments not shown we found that: 1) treatment of endothelial cell monolayers with MBP at concentrations up to 10 μM had no effect on monolayer integrity as assessed morphologically or as quantitated by ^{51}Cr release; 2) virtually identical results were obtained when MBP was not washed out before assay of APC and addition of MBP to APC did not interfere with its detection by chromogenic substrate; and 3) preparations of purified rabbit TM and fibronectin incubated 0.5 h in the presence of high concentrations of MBP showed no evidence of proteolytic digestion by PAGE. In addition to MBP, two other cationic EO granule proteins, EPO and ECP (represented, respectively, by the *open boxes designated EPO and ECP*) also attenuated thrombin-dependent APC generation by porcine endothelial monolayers, in the case of EPO even more potently than MBP. In contrast, BSA at these concentrations had no effect upon APC generation by endothelial monolayers. Thus, the three major cationic proteins comprising EO-specific granules are all potent inhibitors of TM-dependent APC generation by endothelial monolayers.

To assess the durability of MBP inhibition of endothelial monolayer TM function as measured by APC generation and its potential reversibility, we assayed APC generation by monolayers previously treated with nearly 100% inhibitory concentrations of MBP, thoroughly washed, then further incubated in complete tissue culture medium including 10% FCS in either the presence or absence of 10 μg/ml cycloheximide to inhibit protein synthesis. Fig. 3 shows the results of two such experiments, the first using 3.3 μM MBP and the second 5 μM MBP. In the first experiment, MBP-treated endothelial monolayers without cycloheximide (■) regenerate 30% of their capacity to activate protein C 2 h after MBP washout, increasing to 46% by 5 h and 88% after 16 h. By contrast, MBP-treated monolayers incubated in the presence of cycloheximide (▲) recover nly 10% of their activity by 5 h. Over this period cycloheximide has no significant effect upon the APC generation capacity of

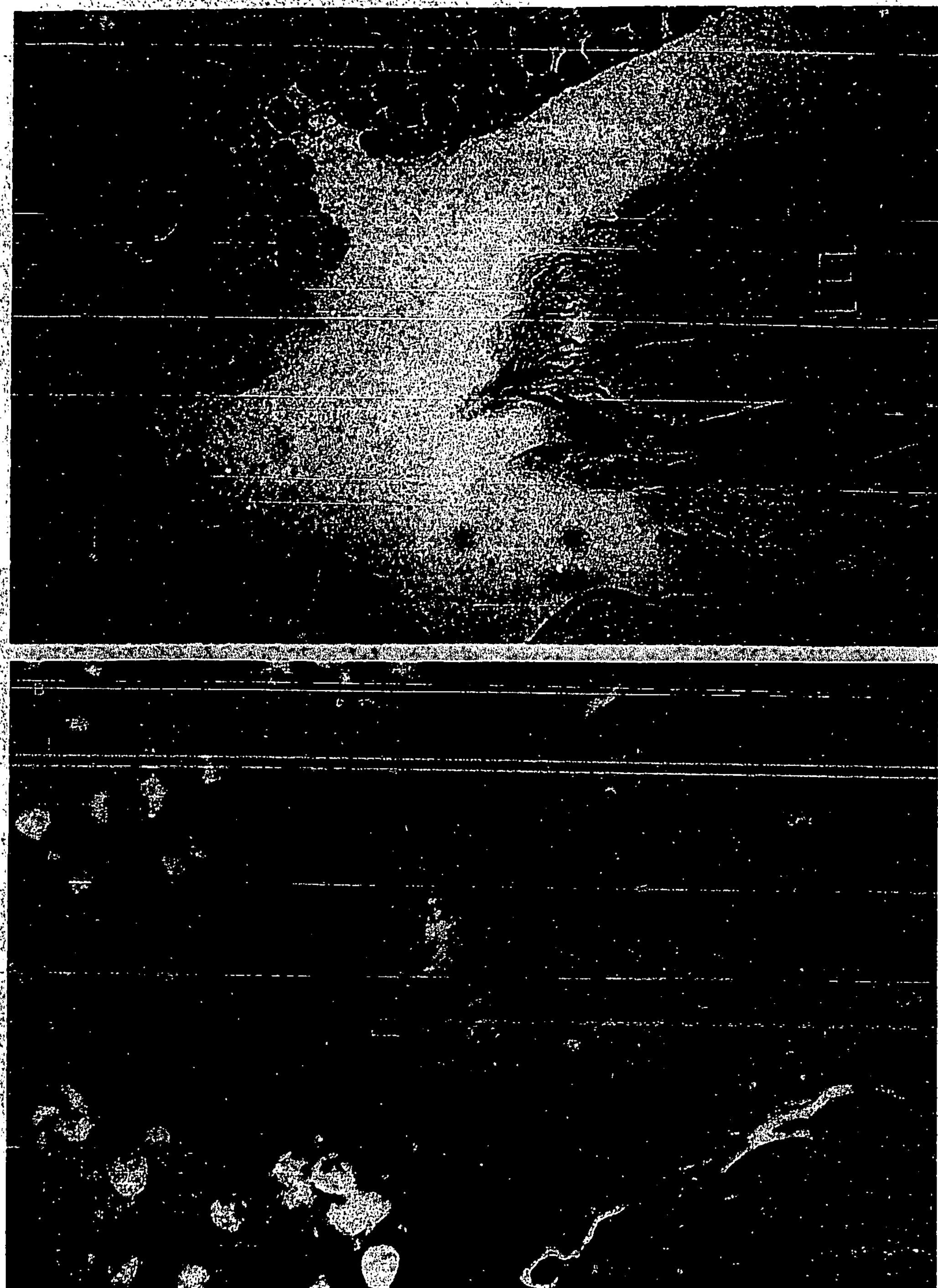

Figure 1. Immunofluorescent localization of MBP in the endocardium of a patient with eosinophilic endocarditis. (*A*) Hematoxylin-and-eosin section of right atrium: ×400. *E*, endocardium; *C*, clot comprised of proteinaceous material and intact EOs. (*B*) Serial section stained for presence of MBP using indirect immunofluorescence. ×400.

monolayers not treated with MBP. By 16 h, however, the cycloheximide-treated preparations are nonviable, thus making meaningful comparisons impossible. In a second experiment performed using 5 μM MBP, monolayers incubated in the absence of cycloheximide (□) recover more slowly and less completely than in the first experiment, but cycloheximide-treated monolayers (Δ) remain nearly completely inhibited through 8 h. Thus, inhibition of endothelial surface TM activity resulting from a single exposure to MBP lasts ≥ 8 h in the absence of protein synthesis; however, partial or even complete reexpres-

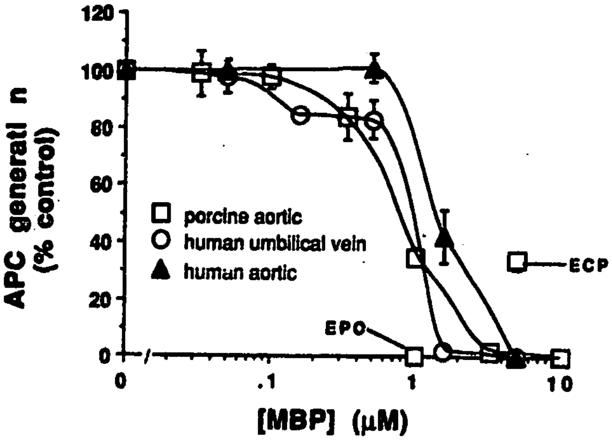

Figure 2. Impaired APC generation by endothelial monolayers exposed to cationic EO granule proteins. 2-cm² endothelial cell monolayers of the indicated derivation were exposed 30 min to 200 μL of H/H buffer supplemented with the indicated concentrations of MBP, thoroughly washed, and assayed for their capacity to support thrombin-dependent activation of protein C in the presence of 1 mM calcium, 500 nM protein C, and 1.5 nM thrombin over 90 min at 37°C. APC was then quantitated using the chromogenic substrate S-2366, as described in Methods. □, porcine aortic endothelium; ○, human umbilical vein endothelium; ▲, human aortic endothelium. For porcine aortic endothelium only, data are shown for single concentrations of ECP and EPO in the open boxes so labeled. Data are shown ±standard deviation.

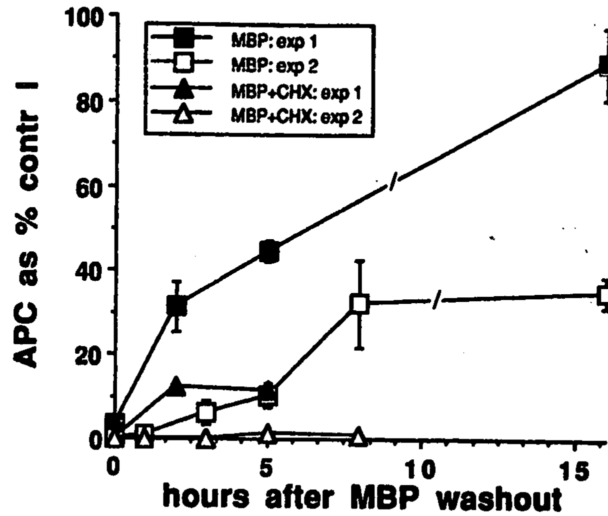

Figure 3. Recovery of APC generation capacity by porcine aortic endothelial cells: time course and requirement for intact protein synthetic capacity. Results of two experiments are shown. In the first, 1-cm² monolayers of porcine aortic endothelial cells were exposed 90 min to 3.3 μM MBP (■) or 3.3 μM MBP with 10 μg/mL cycloheximide (▲), then thoroughly washed with H/H buffer. One set of monolayers from each treatment group was then assayed immediately for APC generation capacity by incubating 90 min in the presence of 500 nM protein C and 0.25 nM thrombin ($T = 0$ at time of washout). Other sets of monolayers were overlaid with 500 mL of Iscove's DMEM with 15% FCS with (▲) or without (■) cycloheximide (CHX). These monolayers were washed and assayed for APC generation capacity at various time points hours after washout. APC generation is expressed as a percentage of that of control monolayers first exposed to, then further incubated in MBP-free buffers with or without CHX, as appropriate. At 16 h CHX-treated monolayers were nonviable; therefore, data for these points are not shown. In the second experiment, the protocol was identical except that monolayers were treated with 5 μM MBP for 30 rather than 90 min in the absence (□) or presence (△) of CHX. Data are shown ±standard deviation.

sion of surface TM activity occurs in cells with intact protein synthetic capacity.

These results suggest that de novo synthesis and surface expression of TM is required for MBP-treated endothelial monolayers to recover TM function. Two potential mechanisms underlying the initial inhibition by MBP include durable blockade of surface TM activity or endocytosis of cell surface TM, as has been shown to occur in PMA-treated hemangioma cells (36) and TNF-treated (37, 38) endothelial monolayers. To address the latter possibility, we determined whether intracellular hypokalemia induced by exposure of endothelial cells to nigericin and extracellular hypokalemia, a potent inhibitor of endocytosis (32), also blocks MBP inhibition of endothelial cell surface TM function. As shown in Table I, depletion of intracellular potassium using the nigericin-hypokalemic buffer protocol had no effect on the ability of MBP to inhibit endothelial monolayer generation of APC in the presence of thrombin. This result suggests that MBP inhibits endothelial cell surface TM activity by blocking the function of TM in situ rather than by inducing endocytosis.

If EO cationic granule proteins impair APC generation of intact monolayers by interacting directly with TM, then these same proteins should inhibit isolated TM in solution as well. We therefore measured the effect of MBP, EPO, and ECP on APC generation in solutions containing purified full-length rabbit lung TM. As shown in Fig. 4, MBP is a potent inhibitor of APC generation with an IC_{50} of 100 nM. EPO, the toxicity f which is typically ascribed to its peroxidative catalytic activity, is also an effective inhibitor of APC generation despite the absence of any hydrogen peroxide substrate, with an IC_{50} of only 10 nM, 10-fold less than that of MBP. Similarly, ECP inhibits APC generation with an IC_{50} of 5 μM (not shown). Thus, EO cationic granule proteins inhibit APC generation by soluble rabbit TM as well as by intact endothelial monolayers.

Table I. Effect of Hypokalemia on MBP Inhibition of Endothelial Cell Surface TM Function

Endothelial monolayer preparation	APC generated in absence of MBP exposure	APC generated after exposure to 10 μM MBP
	mOD_{405}/min	
Normokalemic	98±3	16±2
Hypokalemic	122±7	17±1

1-cm² confluent monolayers of porcine aortic endothelial cells were either left normokalemic or rendered intracellularly hypokalemic by exposure to 4 μM nigericin and hypokalemic extracellular buffers as described in Methods. Normo- and hypokalemic monolayers were further exposed to, respectively, potassium-containing or -free buffers, each either with or without 10 μM MBP. Monolayers were then washed and assayed for APC-generating capacity over 3 h in the presence of 500 nM protein C and 2 nM thrombin. Values are means±SD.

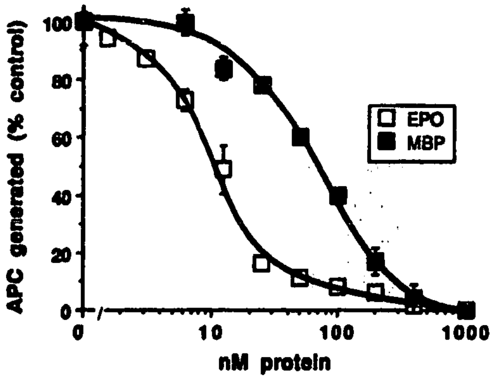

Figure 4. Inhibition of soluble rabbit TM APC generation by MBP and EPO. 2 nM rabbit TM was incubated with the indicated concentrations of cationic protein for 10 min before the addition of 500 nM protein C and 2 nM thrombin at a final calcium concentration of 3 mM, then assayed for APC generation. □, EPO; ■, MBP. 100% = 230 mOD/min at 405 nm. Data are shown ±standard deviation.

To ascertain the mechanism whereby MBP inhibits APC generation by the thrombin/TM complex, we determined the effect of 75 nM MBP (a 65–75% inhibitory dose) on the apparent K_d of rabbit TM for thrombin and the apparent K_m of protein C for the thrombin/TM complex. As shown in Fig. 5 *A*, this concentration of MBP decreases the V_{max} of APC generation with respect to thrombin to approximately one-third that of untreated rabbit TM. Similarly, as shown in Fig. 5 *B*, this concentration of MBP also significantly decreases the V_{max} of APC generation with respect to protein C. These impressions were confirmed by nonlinear kinetic analyses of these data,

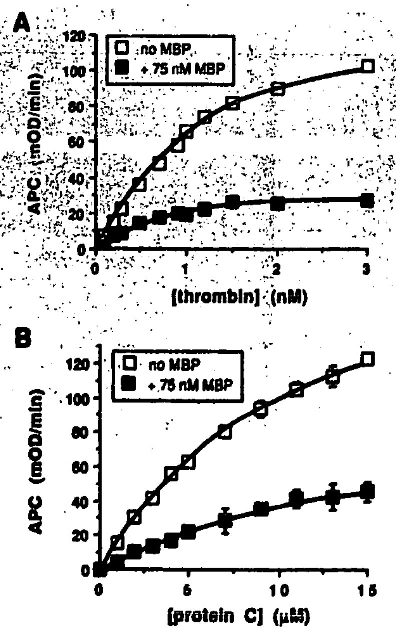

Figure 5. Effect of MBP on thrombin and protein C dependence of protein C activation by rabbit TM. (*A*) Solutions containing 2 nM rabbit TM were incubated in the absence (□) or presence (■) of 75 nM MBP for 10 min before assaying the APC generation in the presence of 3 mM $CaCl_2$ and the indicated concentrations of thrombin. Each data point represents the mean of quadruplicate determinations. Data are shown ±standard deviation. (*B*) Solutions containing 2 nM (final) rabbit TM were incubated in the presence or absence of 75 nM MBP for 10 min before addition of the indicated concentrations of protein C and 0.1 nM thrombin and assayed for APC generation. Each data point represents the mean of triplicate determinations. Data are shown ±standard deviation.

Table II. APC Generation Kinetics of MBP Interaction with TM

Sample	K_d for thrombin	V_{max} for thrombin	K_m (apparent) for protein C	V_{max} for protein C
	nM	*mOD₄₀₅/min*	*μM*	*mOD₄₀₅/min*
Rabbit TM	0.76±0.03	116±2	11.0±2.5	209±26
Rabbit TM +75 nM MBP	0.60±0.05*	31±1*	8.7±3.5	66±19*

Values are means±SD.
* *P* vs. no MBP < 0.05

summarized in Table II. MBP causes a statistically significant but minor decrease in the apparent K_d for thrombin while causing a pronounced decrement of V_{max} to ~ 25% of its control value. 75 nM MBP does not significantly alter the apparent K_m of the thrombin/TM complex for protein C, though our data do not rule out the possibility of a small change. In contrast, the V_{max} of APC generation with respect to protein C is diminished to 31% of its control value in the presence of this concentration of MBP. These data suggest that MBP inhibits APC generation by the rabbit TM complex without materially affecting apparent thrombin binding to TM or protein C binding to the thrombin/rabbit TM complex. Instead, MBP functions primarily as a noncompetitive inhibitor that impairs the catalytic efficiency of the complete thrombin/TM/protein C complex.

We hypothesized that inhibition by EO granule proteins of TM-dependent APC generation reflects an electrostatic interaction between these extremely cationic proteins and the anionic TM molecule, in particular the large O-linked glycosaminoglycan (GAG) moiety located just external to a hydrophobic transmembrane sequence (10–12, 14–20). This unique GAG is comprised of chondroitin sulfate–like dissaccharides, some of which are unusually hypersulfated (and hence more anionic) because they contain two rather than one sulfate per disaccharide unit (14). Because cationic MBP might well bind TM at such a highly anionic site, we predicted that polyanionic substances, and in particular hypersulfated chondroitin sulfates, would reverse MBP-induced inactivation of TM APC generation.

In preliminary experiments not shown, we demonstrated that heparin (3 sulfates/disaccharide), chondroitin sulfate A (1 sulfate/disaccharide), and the hypersulfated chondroitin sulfate E (1.3 sulfates/disaccharide), when present before the addition of MBP, could all effectively block MBP inactivation of rabbit and endothelial cell APC generation. Such apparent blockade might, however, simply reflect polyanion precipitation of cationic MBP, thereby preventing its interaction of TM. We therefore asked instead whether these polyanionic substances could rejuvenate the activity of TM previously inactivated by MBP. As shown in Fig. 6, chondroitin sulfate E and heparin, and, to a lesser extent, chondroitin sulfate A, partially restore the activity of rabbit TM nearly completely inactivated by prior exposure to 200 nM MBP. A hierarchy of efficacy is evident in which heparin > chondroitin sulfate E ≫ chondroitin sulfate A, so that hypersulfated chondroitin sulfate E, which closely resembles the GAG moiety f rabbit TM, more effectively reverses MBP blockade of TM function than does "con-

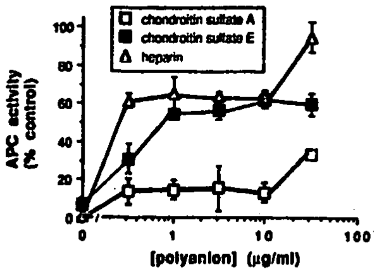

Figure 6. Polyanion reversal f MBP impairment of rabbit TM APC-generating activity. Aliquots f soluble rabbit TM were exposed 10 to 200 nM MBP for 10 min, then incubated further in the presence or absence (buffer control) of the indicated polyanionic substance for another 30 min before assaying APC in the presence of 2 nM thrombin as previously described. □, chondroitin sulfate A; ■, chondroitin sulfate E; △, heparin.

ventionally" sulfated chondroitin sulfate A. The most anionic polysaccharide, heparin, is also the most effective at reversing MBP inhibition of TM.

To investigate directly the role of the TM GAG domain in MBP inhibition of TM function, we used a pair of recombinant human TM mutant proteins, both containing the entire extracellular domain and differing only in the presence or absence of the chondroitin sulfate–like GAG moiety (19, 20, 22). These proteins migrate on SDS-PAGE with apparent molecular masses 105 kD (TMD-105) and 75 kD (TMD-75). Fig. 7 shows the effect of incubating either TMD 105 (GAG^+ form) or TMD 75 (GAG^- form) with increasing concentrations of MBP before assay of APC generation. In the absence of MBP, APC generation was five times as high with TMD-105 as with TMD-75, in agreement with the original description of these isoforms (22). In the presence of MBP concentrations up to 1 μM, APC generation by TM 105 is progressively inhibited with an IC_{50} of ~ 100 nM, similar to that of rabbit TM. In striking contrast, TMD-75 APC generation is unaffected over this same range. At 3.3 and 10 μM, MBP TMD-105 and TMD-75 have nearly identical activities and acceleration is evident. For these paired TM proteins, then, the GAG domain is a prerequisite

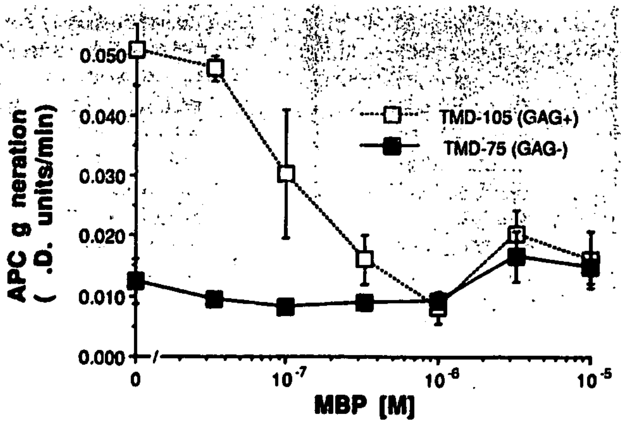

Figure 7. Differential inhibition of APC-generating activity of GAG^+ TMD-105 and GAG^- TMD-75 by MBP. Aliquots of either TMD-105 or TMD-75 (2 nM final) were incubated 5 min in the presence of the indicated concentrations f MBP, then assayed for APC-generating capacity after addition of 2 nM thrombin, as described in Methods. Data shown are ±standard deviation.

for MBP inhibiti n of APC generation. Moreover, the presence of 1–10 μM MBP alters the APC-generating activity of GAG^+ TMD-105 to resemble that of GAG^- TMD-75, as though the functional influence of the GAG domain on APC generation has been negated.

Because cationic EO granule proteins are potent inhibitors of APC generation by the thrombin/TM complex, we determined whether these same proteins could also abrogate another major anticoagulant action of TM, termination of the fibrinogen-cleaving activity of thrombin. As shown in the top two lines of Table III, addition of 20 nM TM lengthened the thrombin clotting time threefold in the case of rabbit TM and fivefold in the case of TMD-105. As expected, the GAG-domainless variant TMD-75 produced only a slight prolongation of the thrombin clotting time (22). The addition of 0.5 or 1 μM MBP alone had only a minor effect on the clotting time. However, when either rabbit TM or TMD-105 was first incubated with these same concentrations of MBP, its ability to prolong the thrombin clotting time was severely curtailed. In the case of TMD-105, MBP treatment resulted in a slight clotting time prolongation nearly identical to that caused by TMD-75 in the absence of MBP. The minor prolongation of clotting time induced by TMD-75 was also partially reversed by exposure to MBP, but to an extent only slightly greater than that attributable to the effect of MBP itself. Identical results were obtained using 300 nM EPO instead of MBP (not shown).

Discussion

These studies demonstrate that three cationic EO granule proteins known to accumulate on endocardial and endothelial surfaces in eosinophilic endocarditis, MBP, EPO, and ECP, all potently inhibit the capacity of endothelial cell monolayers to

Table III. MBP Reversal of TMB-mediated Prolongation of Thrombin Clotting Time

	Clotting time		
Preparation	Rabbit TM	TMD-105	TMD-75
Control	39±1	28±1	28±1.2
+20 nM TM	127±5	142±6	35±1.1
+MBP	48±5	25±1	25±0.6
+TM + MBP	40±2	35±1	29±1.0

Thrombin clotting times were measured using final concentrations of 5 nM bovine thrombin and 2 mg/ml bovine fibrinogen as measured in a fibrometer in H/H buffer supplemented with 0.15% Lubrol PX, pH 7.4. The ability of the designated forms of TM to prolong the thrombin clotting time was assayed by preincubating thrombin 10 min in the presence of 20 nM (final) TM before combining the mixture with fibrinogen to initiate the clotting time. Control clotting times differ between rabbit and the two mutant human TMs because they were assayed in two separate experiments. Where indicated, TM was first incubated 10 min in the presence of MBP (0.5 μM for rabbit TM and 1 μM for TMD-105 and TMD-75) before addition of thrombin, and assay of the thrombin clotting time. Values are means±SD of at least triplicate determinations.

support thrombin-dependent activation f protein C. Such inhibiti n is not likely a result of internalization or endocytosis of surface TM, as apparently happens in the presence of PMA (36) or tumor necrosis factor (37, 38), because it occurs rapidly, within 10–30 min (Fig. 2) rather than over 12–18 h, and intracellular hypokalemia induced by nigericin and hypokalemic buffers, a known inhibitor of endocytosis, does not block it (Table II). In addition, in experiments not shown we find that 1) after exposure to 10 μM MBP < 10% of the original APC-generating activity of endothelial cell monolayers is detected in scraped monolayers disrupted by ultrasonication and assayed for total (i.e., surface and intracellular) APC-generating activity and 2) MBP impairs by 70% the total APC generation of endothelial monolayers metabolically inhibited by maintaining them at 4°C. EO granule protein–mediated inhibition of TM activity is not due to an irreversible toxic effect of these proteins since such inhibition occurs at sublytic concentrations (as detected by ^{51}Cr-release assay), can fully reverse > 16 h after washout of the cationic proteins (Fig. 3), and is not accompanied by release of active TM into supernatant fluid (not shown). Immunohistochemical localization of endothelial cell TM after MBP exposure would help establish whether TM is internalized after exposure to MBP. We have attempted such studies using polyclonal goat anti–porcine TM antibodies but have been thwarted by problems related to nonspecific binding of antibodie: :o MBP-treated monolayers. We therefore conclude that MBP inhibition of endothelial TM function reflects, at least in part, a direct and durable interaction with TM on the cell surface but cannot rule out the possibility that internalization of TM may also play a role in this phenomenon.

This conclusion is further supported by the demonstration that MBP, EPO, and ECP also inhibit APC generation by purified rabbit TM in solution (Fig. 4). EPO and MBP are particularly effective in this regard with IC_{50}s of, respectively, 100 and 10 nM, both roughly 1 μg/ml, well within the range of concentrations of MBP known (35) to circulate in hypereosinophilic individuals. The differing IC_{50}s of MBP for inhibition of endothelial TM as opposed to soluble rabbit TM may be attributable to such factors as differences in species, glycosylation status, and conformation due to cellular TM being expressed in the context of a complex extracellular matrix and membranous surface environment. In comparing the potency of MBP TM inhibition with that of other cationic substances, Preissner et al. (18) have reported that the IC_{50} with regard to APC generation for the synthetic polycation polybrene was 100 μg/ml, for poly-L-lysine 3 mg/ml, and for "platelet releasate" 100 μg/ml. On the other hand, Bourin et al. (17) found little or no effect of the heparin-neutralizing proteins histidine-rich glycoprotein and S-protein in concentrations up to 1,000 nM. This pronounced variance in the ability of cationic proteins to inhibit APC generation suggests factors other than cationicity alone must play a role in this phenomenon. One such factor might be the presence in MBP (39) and EPO (26) of closely juxtaposed cationic and hydrophobic amino acid sequences, a motif common to a variety of membrane-perturbant toxins and venoms (40). In any case, EO granule proteins are the most potent cationic inhibitors f TM APC generation yet described.

MBP inhibits APC generation by rabbit TM not by increasing the apparent K_d for thrombin or the apparent K_m of protein C for the thrombin/TM complex, but rather by acting primarily as a noncompetitive inhibitor. Thus, MBP apparently does not interfere with the binding of thrombin to TM, known to occur in TM epidermal growth factor–like domains 5, and 6, (41, 42) or with binding of protein C to the thrombin/TM complex, activation of which requires epidermal growth factor–like domain 4 (42, 43). Instead, MBP binds to TM electrostatically, perhaps to the GAG domain (see below), and the presence of TM of this intensely cationic substance may distort the normal conformation of the thrombin/TM/protein C complex so as to diminish its catalytic efficiency. Posttranslational γ-carboxylation of protein C does not appear to be critical in MBP-mediated inhibition of rabbit TM function, because the IC_{50} for MBP inhibition of APC generation using Gla-domainless protein C at 3 mM $CaCl_2$ is identical to that for native protein C (data not shown).

MBP-mediated inhibition of TM APC generation is due to a reversible, presumably electrostatic interaction with the TM molecule because the activity of MBP-inactivated rabbit TM is substantially regenerated by subsequent exposure to polyanions such as chondroitin sulfate E, heparin, and chondroitin sulfate A. In considering potential binding sites for MBP on TM, we note that Bourin et al. (14) have demonstrated that the large O-linked GAG moiety attached to the extracellular domain of TM is comprised of an unusually hypersulfated, chondroitin sulfate E–like moiety. That chondroitin sulfate E (1.3 sulfates/disaccharide unit) reverses MBP inactivation of rabbit TM much more effectively than does chondroitin sulfate A (1 sulfate/disaccharide unit) (Fig. 6) suggests that MBP binds TM with an affinity roughly equal to that of MBP for chondroitin sulfate E and is thus compatible with MBP binding to the GAG moiety. Alternatively, the greater negative charge of the chondroitin sulfate E and heparin could equally well disengage MBP from the TM molecule no matter where it were bound.

However, our experiments with TMD-105 (GAG^+) and TMD-75 (GAG^-), paired recombinant human TM proteins that differ only in the presence or absence of the GAG moiety, directly implicate the GAG in mediating the interaction of MBP and TM in two ways. First, whereas both intact rabbit TM and TMD-105 are inhibited by MBP with an IC_{50} of ~100 nM, TMD-75 is relatively unaffected by the presence of MBP (Fig. 7). Thus, presence of the GAG moiety is a prerequisite for MBP inhibition of the APC-generating capacity of TMD. Moreover, at concentrations of MBP > 10^{-6} M the activities of TMD-105 and TMD-75 are identical, suggesting that at these higher concentrations MBP obliterates any influence of the GAG moiety upon TM activity, and thereby renders it in effect functionally identical to TM-75. Second, in addition to impairing APC generation by TM, EPO and MBP also completely abrogate the capacity of fully glycosylated TM to prolong the thrombin clotting time (Table III), as do other cationic proteins (16–18, 21). Others have previously noted that the ability of TM to prolong the thrombin clotting time is strongly influenced by presence of the GAG moiety, because its removal by chondroitinase ABC severely restricts (18, 22) the capacity of TM to perform this function. Our data confirm the observation (22) that GAG^+ TMD-105 (and rabbit TM) are much more effective than GAG^- TMD-75 in this regard and further demonstrate the differential sensitivity of these related forms of TM to inhibition by MBP. As with APC generation, the presence of MBP converts GAG^+ TMD-105 to function as GAG-TMD-75. That is, in the presence of MBP, TMD-105, alone very effective, prolonged the thrombin clotting time to

the same min r extent as TMD-75 does in the absence of MBP. In aggregate, these data suggest that functional inhibition of soluble TM by ECPs is primarily mediated through interactions with the anionic GAG moiety, interactions that mitigate its influence on TM anticoagulant acti ns.

Based on these findings, we propose that in hypereosinophilic states ECPs accumulate in endocardium and endothelial surfaces, bind electrostatically to anionic TM, impair its anticoagulant functions, and thereby promote thrombosis. We recognize, however, that our short-term studies, performed in the absence of plasma, may not accurately reflect in vivo conditi ns in a person with high grade eosinophilia. MBP, for example, circulates in part as a mixed disulfide linked to serum proteins (35) so that free concentrations are lower than the micromolar concentrations of total MBP actually measured (35) in the serum of such persons. Nonetheless, we find that MBP inhibits endothelial cell and soluble rabbit TM APC generation even in the presence of 33–50% serum, although, as might be expected, the IC_{50} are significantly higher at, respectively, 75 and 25 μM. Perhaps more directly pertinent to the role of MBP in vivo is the finding that immunofluorescent studies (reference 8 and Fig. 1) uniformly demonstrate high concentrations of MBP (in fact, concentrations approaching those of intact EO granules) on endocardial and endothelial surfaces of patients with eosinophilic endocarditis. Such accumulation of ECPs may occur either as the result of degranulation of attached EOs or, alternatively, from progressive adsorption of circulating free MBP over weeks or months, a situation difficult to replicate in vitro. Of potential relevance in this regard is the finding that MBP inhibition of endothelial monolayer TM function cannot be reversed by subsequent exposure to buffers containing physiological concentrations (40 mg/ml) of BSA (data not shown). Therefore, MBP, once bound to endothelial TM, is tenaciously attached and cannot be dislodged even by the most anionic and abundant serum protein.

The prominent thromboembolic diathesis that characterizes eosinophilic endocarditis is likely a complex phenomenon ascribable to mechanisms in addition to ECP impairment of TM function. For example, tumor necrosis factor–α, a cytokine that exhibits greatly elevated serum levels in parasitic infestations associated with chronic eosinophilia (44) increases procoagulant tissue factor (37) and decreases TM expression in vascular endothelium (37, 38), and, as we have previously shown, renders endothelium more vulnerable to damage by activated EOs (45). ECP accelerates coagulation through a Factor XII–dependent mechanism (46). Moreover, both MBP and EPO evoke nonlytic platelet secretion of serotonin, α-granule, and lysosomal proteins with an EC_{50} of 20–30 μg/ml (47), a range similar to that in which we demonstrate inhibition of TM-dependent APC generation by intact endothelial monolayers (Fig. 2). Finally, subtle damage to endothelial cell surfaces unrelated to TM and to extracellular matrix wrought by reactive oxygen intermediates or ECPs may render these surfaces procoagulant by a variety of potential mechanisms.

In addition to providing potential insights into the thromboembolic diathesis that attends hypereosinophilic heart disease, our studies further emphasize the importance of the GAG domain in TM function, suggest the potential usefulness of cationic proteins as tools to study TM structure–function relationships, and invite further examination of other potentially relevant cationic proteins that might modulate of TM function. With regard to the second possibility, we have found in preliminary experiments that MBP appears to unmask a high affinity calcium-binding site n TMD-105, converting its APC-generating calcium concentration optimum to resemble that of TMD-75 (22), chondroitin ABC lyase–cleaved TMD-105 (20), and elastase-cleaved rabbit TM (48), with maximum activity at subphysiologic (0.3 mM) calcium concentrations. Finally, given the relative potency of "platelet releasate" as an inhibitor of TM-dependent APC generation (18) and the pronounced similarities of platelet Factor 4 and MBP with regard to cationicity, heparin binding, and inhibition of angiogenesis (49, 50), it will be of great interest to see if platelet Factor 4 or some other cationic component released by activated platelets also inhibits TM function of endothelial monolayers, a finding of obvious physiologic as well as pathologic relevance.

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Idiotope Determining Regions of a Mouse Monoclonal Antibody and Its Humanized Versions

Identification of Framework Residues that Affect Idiotype Expression

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The contribution of framework regions (FRs) of antibody-variable domains to idiotype expression was studied by examining the interaction of various "humanized" versions of a mouse anti-TNFα monoclonal antibody (mAb78) with polyclonal and two monoclonal antibodies (mAb1G3 and mAb9F1), generated against the mAb78 idiotype.

Humanized mAb78, bearing human constant domains and mouse complementarity-determining regions (CDRs) inserted with human FRs, was found to be five to sevenfold less reactive than mAb78 with polyclonal anti-idiotype antibodies and 200 to 300-fold less active in neutralizing TNFα. The substitution of heavy-chain FRs residues of the humanized antibody with original mouse residues 28 to 30, 48 to 49, 67 to 68, 70 to 71, 78, 80 and 82 progressively restored the immunoreactivity with polyclonal immunoglobulin Gs to the level of a version having mouse heavy chain and human light chain FRs, and increased 10 to 20-fold the TNFα neutralizing activity. This suggests that at least some of these residues are critical for TNFα binding as well as for the expression of idiotopes that are strongly immunogenic in syngeneic animals.

All antibody versions with either human or mouse FRs were able to bind to various extents mAb1G3, a γ-type anti-Id antibody that inhibits mAb78/TNFα interaction by paratope blockade. At variance, only the antibody versions containing mouse FRs were able to bind mAb9F1, an α-type anti-Id antibody unable to block the access of TNFα to mAb78 paratopes. Substitution of heavy chain FR residues 28 to 30 markedly decreased the binding of mAb1G3 (100 to 1000-fold). This suggests that these antibodies recognize CDR and FR idiotopes, respectively, that can be drastically modified by changes in the FRs.

In conclusion, the results suggest that CDRs as well as FRs markedly contribute to antibody Id expression. Although strongly immunogenic idiotopes are probably located within the CDRs, the results also suggest that some FR residues are critically involved in shaping antibody Id diversity by affecting the structure of CDR-related idiotopes.

Keywords: monoclonal antibody; humanized antibody; chimeric antibody; idiotype; tumour necrosis factor

The composite collection of antigenic determinants associated with the variable (V) domains of immunoglobulins is called idiotype (Id‖). A particular Id can be uniquely expressed by a single clonally derived immunoglobulin (private Id) or can be shared by different immunoglobulins (cross-reac-

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‖ Abbreviations used: Id, idiotype; Ag, antigen; CDR, complementarity determining region; FR, framework region; TNFα, human tumour necrosis fractor-α; BSA, bovine serum albumin; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline sulphonate).

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tive, public or recurrent Id; Paul & Bona, 1982). A network of Id–anti-Id interactions has been proposed to play a role in immunoregulation (Jerne, 1974; Paul & Bona, 1982; Nisonoff, 1991; Rodey, 1992); moreover, anti-Id antibodies have been used as reagents in many research and diagnostic applications as well as for therapeutic purposes (Nisonoff, 1991; Kohler *et al.*, 1989; Rodey, 1992; Poskitt *et al.*, 1991). The topology of idiotypic determinants on antibody V-domains and interconnections with other idiotopes expressed on B and T cell receptors in the idiotypic repertoire is thought to be important for the functional properties of anti-Id antibodies (Bona, 1988; Nisonoff, 1991; Kohler *et al.*, 1989). A number of studies aimed to characterize the structural basis of idiotypy and the idiotope topology have been carried out by low-resolution mapping studies based on competitive binding with antigens and monoclonal anti-Id antibodies (Greenspan & Davie, 1985; Zenke *et al.*, 1985; Streicher *et al.*, 1986), electron microscopy (Roux *et al.*, 1987), amino acid sequence and mutational analysis of Id (reviewed by Greenspan & Bona, 1993) and crystal structure analysis of an Id–anti-Id complex (Bentely *et al.*, 1990). It has been shown that both hypervariable and framework regions of heavy and light chains could contribute to idiotopes. In particular, studies on idiotope topography of one idiotypic system, based on competitive binding with monoclonal antibodies, were suggestive of a linear idiotope map spanning the V-domain from the antigen binding site to the vicinity of constant (C) domain (Greenspan & Davie, 1985).

In this work we have investigated the functional contribution of FRs to Id expression by examining the interactions of anti-Id polyclonal and monoclonal antibodies, developed in syngeneic mice against a mouse anti-TNFα neutralizing antibody (mAb78) (Barbanti *et al.*, 1993; Corti *et al.*, 1993), with a series of humanized mAb78 variants having partial or complete human FRs.

Several humanized variants of mouse antibodies have recently been described: it has been shown that mouse V-domains can be joined with human C-domains, to form mouse–human chimeric molecules, and that mouse CDRs can be transplanted within human FRs without losing antigen (Ag) specificity (Morrison *et al.*, 1984; Boulianne *et al.*, 1984; Neuberger *et al.*, 1985; Jones *et al.*, 1986; Brown *et al.*, 1987; Liu *et al.*, 1987; Verhoeyen *et al.*, 1988; Riechmann *et al.*, 1988; Hale *et al.*, 1988; Queen *et al.*, 1989; Mathieson *et al.*, 1990; Gorman *et al.*, 1991; Tempest *et al.*, 1991; Co *et al.*, 1991; Maeda *et al.*, 1991; Kettleborough *et al.*, 1991). Significant

Table 1

Isotype, FRs and TNFα neutralizing activity of mAb78 versions used for Id expression studies

Version†	Isotype	Residues changed in human FRs‡ H chain	Residues changed in human FRs‡ L chain	TNFα neutral. activity§ (ng/ml ± S.D.)
mAb78	Mouse IgG1,k	All	All	6·7 ± 1·4
MuVH/MuVK	Human IgG1,k	All	All	7·7 ± 2
MuVH/HuVK	Human IgG1,k	All	None	9·7 ± 1·1
HuVH(*SLTLGLRTKVFL*)/HuVK	Human IgG4,k	28, 29, 30, 48, 49, 67, 68, 70, 71, 78, 80, 82, 94	None	110 ± 8·5
HuVH(*SLTLGLRTK*)/HuVK	Human IgG4,k	28, 29, 30, 48, 49, 67, 68, 70, 71, 94	None	163 ± 30
HuVH(*SLT*)/HuVK	Human IgG1,k	28, 29, 30, 94	None	2400 ± 605
HuVH/HuVK	Human IgG1,k	94	None	2200

Experiments for TNFα neutralizing activity measurements. Mouse L-M fibroblasts (ATCC CCL1.2) were cultured in Minimum Essential Medium with Earle's salts (Gibco), 5% foetal calf serum (FCS), 2 mM glutamine (MEM-FCS) at 37 °C, in an atmosphere of 5% carbon dioxide/95% air. Anti-TNFα antibodies were incubated at various concentrations in the presence or absence of 1 ng recombinant TNFα/ml (Esquire Chemie AG, Zurich, Switzerland), for 2 h at 37 °C in MEM-FCS. Each mixture and actinomycin D (final concentration, 2 μg/ml) were then added to mouse L-M cells that had been seeded the day before at 30,000 cells/well in 96-well flat bottom plates in MEM-FCS. After overnight incubation at 37 °C, 5% CO_2, live cells were stained with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (final concentration, 850 μg/ml) for 4 h. Supernatants were then aspirated, dimethyl sulphoxide was added (200 μl/well) and the optical density of each well was read at 570 nm. The concentration of antibody able to give a cell viability corresponding to 50% of controls (without TNFα and with actinomycin D) was taken for comparing the TNFα neutralizing activity of humanized antibodies.

† The preparation and characterization of mouse mAb78 was described previously (Barbanti *et al.*, 1993). Chimeric (mouse–human) and reshaped mAb78 versions were prepared, purified and characterized as described elsewhere. CDR sequences are identical to those of mAb78 in all versions.

‡ Heavy and light chain FR sequences are those of the human antibody HIL and LAY, respectively (Kabat *et al.*, 1991). Numbers indicate residues that have been changed with original mouse residues.

§ Concentration of antibody that neutralizes 50% cytolytic activity of 1 ng TNFα/ml on L-M cells.

losses of affinity and biological potency have been observed in most cases after CDR grafting, even when the human FRs had been selected as the most homologous with the original mouse antibody. However, the affinity and biological potency of reshaped antibodies was restored by introducing original mouse residues in the human FRs at positions known to be involved in antigen binding and/or in maintaining the proper conformation of the CDRs (Tempest *et al.*, 1991; Kettleborough *et al.*, 1991; Foote & Winter, 1992).

Keeping this in mind, to investigate the functional contribution of structural regions and domains of mAb78 (CDRs, FRs and V and C-domains) to Id expression we have examined the Ag-binding properties as well as the Id expressed by the following humanized mAb78 versions (see Table 1): (1) a mouse–human chimeric mAb78 (MuVH/MuVK), consisting of mAb78 mouse V-domains joined with human C-domains; (2) a partially reshaped mAb78, characterized by complete mouse heavy chains and human light chain FRs (MuVH/HuVK); (3) four reshaped mAb78 versions, consisting of mAb78 CDRs inserted within the heavy and light chain V-domain on FRs of the human antibodies HIL and LAY (Kabat *et al.*, 1991), respectively, and bearing increasing numbers of original mouse FR residues thought to be important for antigen binding (Amit *et al.*, 1986; Chothia & Lesk, 1987; Tramontano *et al.*, 1990; Foote & Winter, 1992; Tempest *et al.*, 1991). The human FRs have been selected as the most homologous to the original mouse FRs (P. R. Tempest *et al.*, unpublished results).

The effect of FR changes on antigen binding was investigated first. As shown in Table 1, the TNFα neutralizing activity of HuVH/HuVK was 300-fold lower than that of MuVH/MuVK and original mAb78. Of note, substitution of residues 28 to 30, 48 to 49, 67 to 68, 70 to 71, 78, 80 and 82 of heavy chain FRs with the original mouse residues increased 10 to 20-fold the TNFα neutralizing activity, clearly indicating that these residues are critically involved in TNFα recognition.

The Id expressed by each mAb78 variant was then studied by competitive and direct binding experiments with anti-Id antibodies: competitive ELISA was carried by measuring the binding of mAb78-HRP to solid-phase anti-Id antibodies in the presence of mAb78 versions at various concentrations. Direct ELISA was carried out by measuring the binding of anti-Id antibodies to humanized antibodies adsorbed on microtitre plates through goat anti-human IgGs ("direct" ELISA). This type of adsorption was chosen in order to immobilize antibodies through their C-domains and, therefore, to reduce the risk of artefacts due to a different orientation of antibodies on the solid-phase.

Id expression studies were first carried out with a mixture of affinity-purified polyclonal anti-mAb78 IgGs prepared by immunizing BALB/c mice with mAb78 coupled to cationic BSA, BSA or keyhole limpet haemocyanin (Fig. 1).

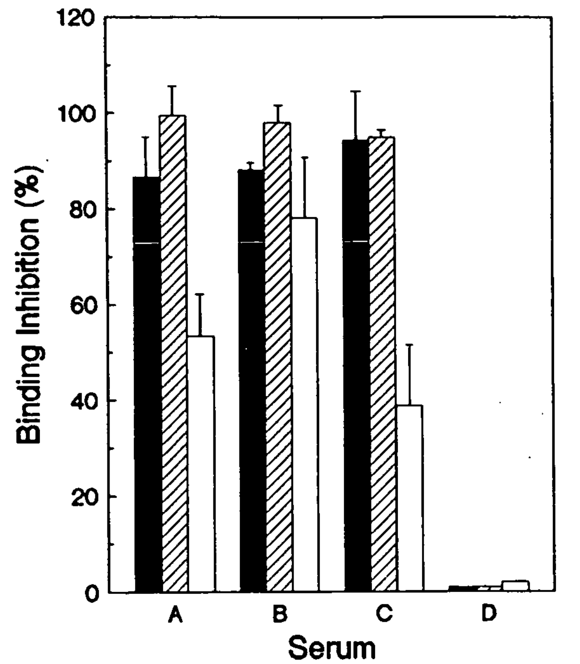

Figure 1. Detection of anti-mAb78 Id antibodies in sera from animals immunized with mAb78 coupled to cationic BSA (cBSA, Pierce) (A); BSA (B); keyhole limpet haemocyanin (C); and in normal mouse serum (D). Syngeneic polyclonal anti-mAb78 antibodies were raised in 9 BALB/c mice by immunizing with each conjugate (3 animals per group), as previously described (Corti *et al.*, 1993). The production of antibodies was checked by measuring the capability of each serum to inhibit the binding of biotin-TNFα to mAb78 as follows. To this purpose, poly(vinyl-chloride) (PVC) microtitre plates (Falcon Microtest III flexible assay plates, Becton Dickinson) were coated with 1 μg mAb78/ml in PBS (0·15 M sodium chloride, 0·05 M sodium phosphate, pH 7·3) (50 μl/well, overnight at 4°C) and blocked with 200 μl of PBS, 3% BSA (2 h at 37°C). After washing with PBS-T (PBS containing 0·05% (v/v) Tween 20), wells were incubated with samples (50 μl, 1 h at 37°C), washed again as above, and incubated with biotinylated TNFα, 1 : 1000 in PBS-T (containing 1% (w/v) BSA) (50 μl/well, 1 h at 37°C). Bound biotinylated TNFα was detected with streptavidin-HRP, 1 : 2000 in PBS-BT (50 μl/well, 1 h at 37°C). After a final washing each well was incubated with 100 μl of 2,2′-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) chromogenic solution (Boehringer-Mannheim) (30 min at 37°C) and the absorbances at 405 nm of each well was measured. As shown, all sera were able to inhibit the binding to a similar extent, suggesting that all conjugates elicited anti-Id antibodies to comparable levels. Thus, all animal sera were pooled and affinity-purified on a mAb78-agarose column as follows: 1 ml of pooled serum was centrifuged at 20,000 *g* for 15 min and loaded onto a mAb78/agarose column prepared by using the "ImmunoPure Ag/Ab Immobilization" Kit (Pierce) (3 mg of mAb78 were coupled to 2 ml of activated-agarose according to the manufacturer's instructions). The flow was stopped and the column incubated overnight at 4°C. The column was then washed with 20 ml of PBS and eluted with a 0·1 M glycine solution (pH 2·8). The absorbance at 280 nm of the effluent was monitored. Peak fractions were pooled, neutralized and stored at −20°C. Antibody concentration was estimated by measuring the absorbance at 280 nm ($E_{1\%}$, 280 nm = 13). Dilutions: 1 : 100 (■); 1 : 1000 (▨); 1 : 10,000 (□).

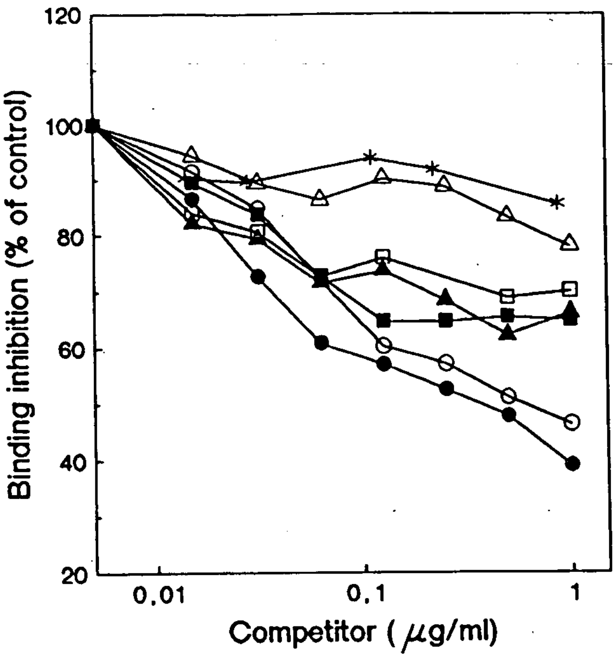

Figure 2. Competitive binding of mAb78 versions and mAb78-HRP to solid-phase polyclonal IgGs. The mAb78-HRP conjugate was prepared as follows: 100 µl of purified mAb78 (3 mg/ml in PBS) was mixed with 100 µl of an HRP solution (10 mg/ml in 0·1 M sodium phosphate buffer, pH 6·5) and with 10 µl of 1 % (v/v) glutaraldehyde. The solution was incubated 4 h at room temperature and mixed with 20 µl of a 1 M lysine solution. After 1 h incubation, the product was dialyzed against 0·1 M sodium phosphate buffer (pH 6·5) and stored as a stock solution at −20°C. Competitive binding experiments were carried out as follows: PVC microtitre plates were coated with a 10 µg/ml solution of anti-Id antibodies in PBS (50 µl/well, 1 h at 37°C) and blocked with 200 µl of PBS, 3% BSA (2 h at 37°C). After washing with PBS-T, wells were incubated with, mAb78 derivatives (humanized versions or peptides) at various concentrations in PBS-BT (50 µl, 2 h at 37°C), mixed 1 : 1 with mAb78-HRP, previously diluted with PBS-BT (1 : 400 of stock solution), and further incubated for 1 h at 37°C. After a final washing with PBS-T, each well was incubated with 100 µl of ABTS chromogenic solution (30 min at 37°C) and the absorbances at 405 nm were measured. For abbreviations, see the legend to Fig. 1. Competitors: mAb78 (●); MuVH/MuVK (○); MuVH/HuVK (■); HuVH (SLTGLRTKVFL)/HuVK (□); HuVH(SLTGLRTK)/HuVK (▲); HuVH(SLT) HuVK (△); HuVH/HuVK (✱).

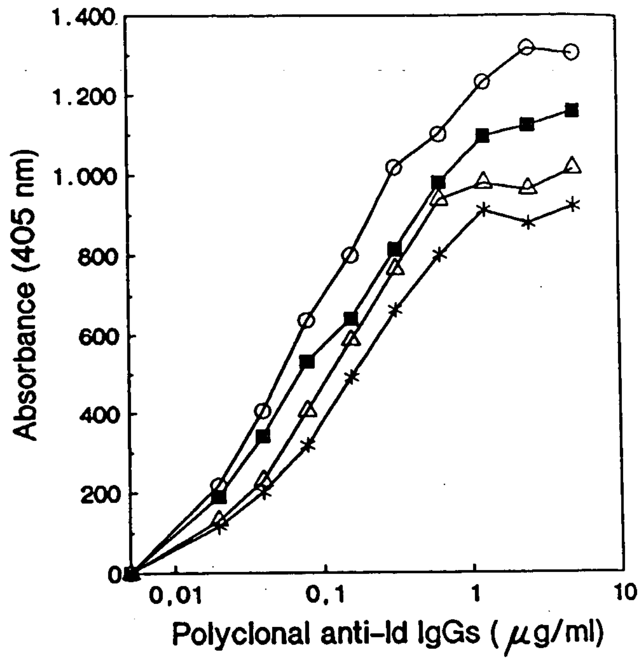

Figure 3. Binding of polyclonal anti-Id IgGs to solid-phase mAb78 versions. PVC microtitre plates were coated with a goat anti-human IgG solution (40 µg/ml, in PBS, 50 µl/well, overnight at 4°C) and blocked with PBS, 3% BSA (200 µl/well, 2 h at 37°C). After washing with PBS-T, wells were incubated with 50 µl of humanized antibody solution (100 ng/ml in PBS-BT, 1 h at 37°C). The plates were washed again with PBS-T and incubated with anti-Id antibody solutions at various concentrations, in PBS-BTGH (PBS-BT containing 1 % (v/v) normal goat serum and 1% (v/v) normal human serum (50 µl/well, 1 h at 37°C), After washing with PBS-T each well was incubated with a goat anti-mouse IgG-HRP conjugate solution (1 : 1000 in PBS-BTGH, 50 µl/well, 1 h at 37°C) and washed further with PBS-T. Each well was then incubated with 100 µl of ABTS chromogenic solution (30 min at 37°C) and the absorbances at 405 nm were measured. For abbreviations, see the legend to Fig. 1. Solid phases: MuVH/MuVK (○); MuVH/HuVK (■); HuVH(SLT)/HuVK (△); HuVH/HuVK (✱).

The results of competitive and direct binding assays (Figs 2 and 3) showed that joining of mAb78 V-domains with human C-domains (IgG1 and kappa) essentially preserves the mAb78 idiotype, as suggested by similar immunoreactivity of mAb78 and chimeric MuVH/MuVK. In contrast, extensive humanization of mAb78 V-domains was found to markedly affect the Id expression, as the HuVH/HuVK version was five to sevenfold less reactive. The substitution of human heavy chain FR residues of the HuVH/HuVK with the original mouse residues 28 to 30, 48 to 49, 67 to 68, 70 to 71, 78, 80 and 82 progressively increased the immunoreactivity with polyclonal IgGs to the level of the MuVH/HuVK version. Since this version bears murine heavy chains, the results suggest that the CDRs and at least some of these residues are critically involved in shaping the mAb78 heavy chain idiotype. Moreover, as these residues are also critical for TNFα binding (Table 1), it is very likely that strong idiotypic determinants of mAb78 are located within or close to the CDRs.

The idiotype of humanized mAb78 versions was further studied with monoclonal antibodies. In previous works we have described the production and characterization of two monoclonal anti-Id antibodies against mAb78 (mAb1G3 and mAb9F1), able to recognize non-overlapping heavy chain idiotopes located within and outside the paratope,

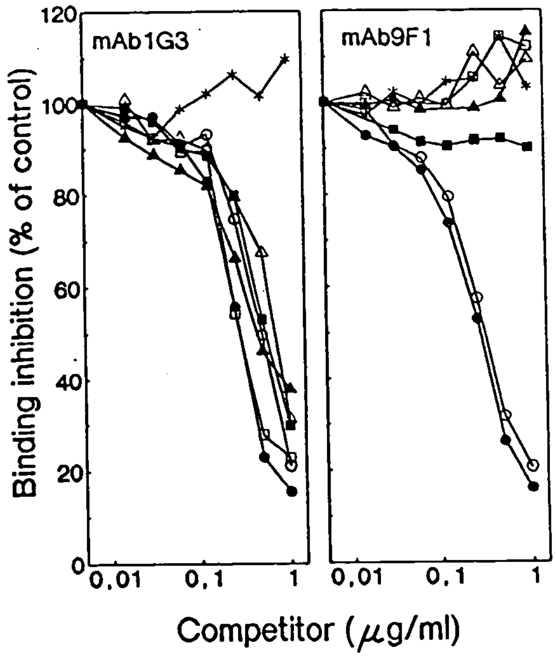

Figure 4. Competitive binding of mAb78 versions and mAb78-HRP to solid-phase mAb1G3 (left) and mAb9F1 (right), measured by "competitive" ELISA as described in the legend to Fig. 2.

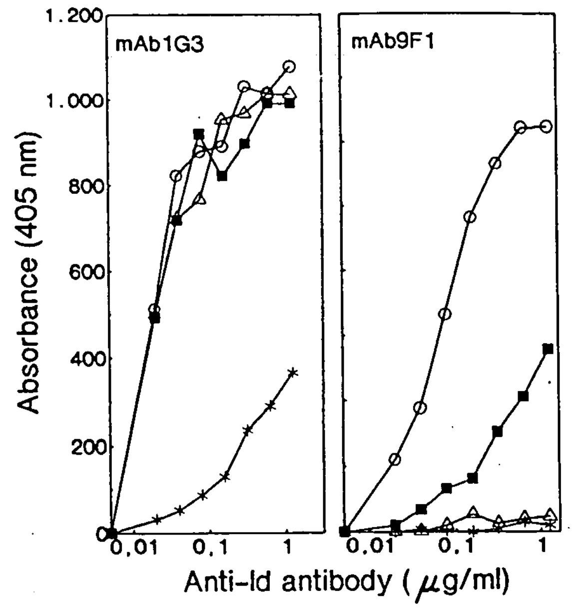

Figure 5. Binding of mAb1G3 (left) and mAb9F1 (right) to solid-phase mAb78 versions, measured as described in the legend to Fig. 3.

respectively (Corti *et al.*, 1993; Barbanti *et al.*, 1993). Thus, mAb1G3, according to Bona & Kohler (1984), is a β or γ-type anti-Id mAb, whereas mAb9F1 is an α-type antibody.

Previously we have also shown that mAb9F1 is able to inhibit the TNFα/mAb78 interaction in a non-competitive mode by affecting the avidity of bivalent mAb78 for TNFα oligomers, whereas mAb1G3 is able to inhibit the interaction in a competitive mode by paratope blockade.

The mAb1G3-defined idiotope was studied first. The binding of mAb78-HRP to solid-phase mAb1G3 (Fig. 4, left panel) was efficiently competed by mouse and chimeric mAb78 as well as by all reshaped antibodies having the mouse residues Ser28, Leu29 and Thr30 in the FRs. At variance, no competition was observed with a reshaped mAb78 version (HuVH/HuVK) having the human FR residues Thr28, Phe29 and Ser30. Nevertheless, a weak interaction was also observed with HuVH/HuVK in a direct binding assay, this analytical system being more sensitive than the competitive assay (Fig. 5, left panel). In this system, mAb1G3 was found to bind the HuVH(SLT)/HuVK version with an affinity apparently close to that of mAb78, while it bound the HuVH/HuVK version with a 100 to 1000-fold lower affinity. These results indicate that mAb1G3 is able to recognize all antibody versions with either human or mouse FRs and that the heavy chain FR residues 28 to 30 (SLT) are critical for full idiotope expression.

The FR residues 28 to 30 are part of the H1 hypervariable loop (residues 26 to 32) defined by Chothia & Lesk (1987). From computer graphic models of mAb78 VH, with Thr28, Phe29, Ser30 or with Ser28, Leu29, Thr30, the side-chains of residues 28 and 30 appear to be located on the surface whereas the side-chain of residue 29, facing inward and buried within the FR structure, appears to make important interactions that may affect the conformation of CDR 1 and 2 (not shown). It is therefore possible that these residues are either a part of the mAb1G3 idiotope or are necessary to support the conformation of an idiotope located on CDRs.

To evaluate if the residues 28 to 30 are directly involved in mAb1G3 binding, a peptide corresponding to mAb78[25–33] residues (SGFSLTGYG), comprising the entire H1 loop, was synthesized and tested by "competitive" ELISA in parallel with mAb78. While 50% binding inhibition was observed with about 250 ng mAb78/ml, the mAb78(25–33] peptide was unable to compete even at a 1000-fold higher concentration. Moreover, mAb1G3 was unable to bind the mouse myeloma protein MOPC141, a member of the Kabat subgroup IB as mAb78, containing the same 25 to 33 residues (Kabat *et al.*, 1991, our results not shown). This favours the hypothesis of an indirect role of these residues in determining the expression of an idiotope located on CDRs, though it cannot be excluded that they are also part of a strongly conformational idiotope close to the paratope.

The anti-paratypic nature of mAb1G3 was investigated further. It has been reported by several authors that, in some cases, anti-paratypic antibodies may mimic some of the immunological and biological properties of antigens. These antibodies have been classified as β-type and are also called

"internal images" (Farid & Linthicum, 1988; Bona, 1988). Interestingly, mAb1G3 binds an idiotope located close to the paratope, by antigen-binding inhibition studies (Corti *et al.*, 1993) and is able to cross-react with anti-TNFα polyclonal antibodies raised in syngeneic animals (Barbanti *et al.*, 1993). However, we found that HuVH/HuVK and HuVH/(SLT)/HuVK are able to neutralize TNFα with similar potency (Table 1) and bind TNFα with similar efficiency in ELISA (Fig. 6), although the affinities of these versions for mAb1G3 are markedly different (Figs 4 and 5). Hence, three FR substitutions that do not affect antigen binding, affect the expression of the mAb1G3-defined idiotope. This suggests that idiotope and paratope are different and implies that mAb1G3 is a γ-type anti-Id mAb. Accordingly, we were unable to raise anti-TNFα antibodies by injecting mAb1G3 in syngeneic animals, as expected for a β-type antibody (results not shown). Therefore, the capability of mAb1G3 to recognize anti-TNFα polyclonal antibodies raised in a syngeneic animal, previously observed (Barbanti *et al.*, 1993), is more likely related to binding to a recurrent idiotype (Paul & Bona, 1982).

The mAb9F1-defined idiotope was then studied. As shown in Figures 4 and 5, mAb9F1 was able to bind only the antibody versions containing complete mouse heavy chain FRs. Though mAb9F1 recognizes an idiotope apparently located on the heavy chain by Western blot analysis (Corti *et al.*, 1993) this antibody was able to bind the MuVH/HuVK version very weakly, suggesting that pairing of both heavy and light chain mouse FRs is necessary for full expression of the mAb9F1 idiotope. These findings and the previous observation that mAb9F1 is unable to inhibit TNFα binding to monovalent mAb78 Fab fragments (Corti *et al.*, 1993), strongly suggests that this antibody recognizes an α-type idiotope located on FRs. Thus, mAb1G3 and mAb9F1 may be representative of two subpopulations of polyclonal anti-Id IgGs against γ and α-type idiotopes, respectively: the first able to recognize CDR-related idiotopes and the second able to recognize FR-related idiotopes, both drastically modified by changes in the FRs.

In conclusion, in this work we have shown that CDRs as well as FRs, but not C-domains, contribute markedly to mAb78 Id expression. This is in agreement with the concept that both CDRs and FRs contribute to Id expression of immunoglobulins (Greenspan & Bona, 1993). Moreover, the evidence for the location of mAb9F1 idiotope on the FRs is also consistent with studies demonstrating that the entire V-domain surface, stretching from the CDR to the V-C junction, may express idiotopes (Greenspan & Davie, 1985; Roux *et al.*, 1987). However, the finding that strongly immunogenic mAb78 CDR-related idiotopes recognized by polyclonal and monoclonal antibodies may be drastically modified by changes of a few FR residues critical for TNFα binding, suggests that the CDR surface expresses immunodominant idiotopes, in syngeneic animals, and that the FRs are critically

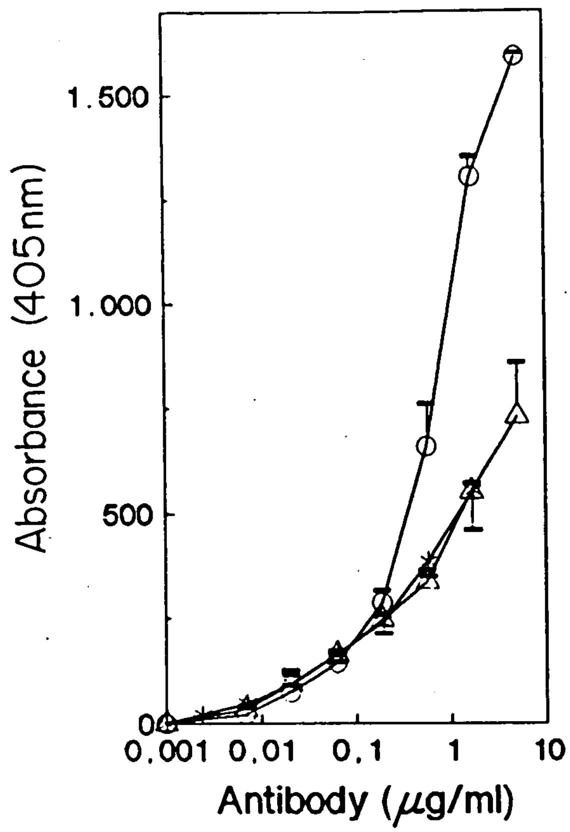

Figure 6. Binding of mAb78 versions to TNFα. PVC microtitre plates were coated with a TNFα solution (1 μg/ml, in 0·1 M sodium carbonate buffer (pH 9·6), 50 μl/well, overnight at 4°C) and blocked with PBS, 3% BSA (200 μl/well, 2 h at 37°C). After washing with PBS-T, wells were incubated with 50 μl of mAb78 versions at various concentrations in PBS-BT (1 h at 37°C). The plates were washed again with PBS-T and incubated with a goat anti-human IgG-biotin conjugate solution (1:800 in PBS-BT containing 1% normal goat serum, 50 μl/well, 1 h at 37°C). The plates were washed with PBS-T and filled with a 1:2000 streptavidin-HRP solution in PBS-BT containing 1% normal goat serum (50 μl/well). After 1 h incubation and a final washing with PBS-T, each well was incubated with 100 μl of ABTS chromogenic solution (30 min at 37°C) and the absorbances at 405 nm were measured. For abbreviations, see the legend to Fig. 1. MuVH/MuVK (○); HuVH(SLT)/HuVK (△); HuVH/HuVK (✱).

involved in shaping antibody Id mainly by affecting the CDR structure.

CDRs and FRs of antibody V-domains have been defined on the basis of homology between known primary structures of immunoglobulins (Kabat *et al.*, 1991). X-ray crystallographic studies have shown that the CDRs of each chain include hypervariable loops involved in antigen binding and connecting the strands of two layers of β-pleated sheets that form the V-domain scaffold (Poljak *et al.*, 1973; Schiffer *et al.*, 1973; Segal *et al.*, 1974; Chotia & Lesk, 1987). Accordingly, transplantation of mAb78 CDRs onto the HIL FRs was sufficient to retain the TNFα specificity. Previous studies on

immunoglobulin structures suggest that the FR residues identified in this work, critical for Id expression and TNFα binding, could be critical for determining the structure of the hypervariable loops: for instance, the heavy chain residues 28 and 30 are surface-exposed and structurally part of the loop incorporating CDR 1 (Amit *et al.*, 1986), while the side-chains of residues 29 and 71 face inward and play a role in packing and determining the conformation of CDRs 1 and 2 (Chothia & Lesk, 1987; Tramontano *et al.*, 1990). Residues 48, 49, 67 and 78 are part of the FR residues forming a layer underlying the CDRs that may finely adjust the CDR structure (Foote & Winter, 1992). Moreover, residues 68, 70, 80 and 82 form hydrogen bonds to adjacent β-strands which form the scaffold for loop disposition (Chothia & Lesk, 1987).

It is very likely, therefore, that these FR residues could contribute to Id expression by affecting the loop structure. In addition, it is also possible that some of the structural changes induced by substitution of these FR residues affect the interplay occurring between heavy and light chains that could influence the proper juxtaposition and orientation of hypervariable loops and, consequently, the overall CDR structure.

Kieber-Emmons & Kohler (1986) have described 5 heavy chain and 6 light chain regions characterized by high surface variability, as identified by comparing the variability plots and hydropathic profiles of a number of human and mouse antibody V-domains. Since surface accessibility and variability are known to be important features of epitopes in proteins, these regions were thought to have the intrinsic potential to express idiotypic determinants and, therefore, were called idiotope-determining regions (IDR). This concept is well supported by our finding that the residues 28 to 30, 48 to 49 and 70 to 71 belonging to the IDR-A[27–31], IDR-B[48–53] and IDR-D[70–75], respectively, are important for the expression of idiotopes recognized by polyclonal and monoclonal anti-Id IgGs, and that CDRs, partially overlapping with the proposed IDRs, are likely to express immunodominant idiotopes.

It has been proposed that a network of Id–anti-Id interactions contributes to the regulation of the immune response (Jerne, 1974; Paul & Bona, 1982; Nisonoff, 1991; Rodey, 1992): in view of this hypothesis it is possible that variability at some of the FR sites identified in this work within the primary repertoire and in affinity maturation play a role in the generation of structural diversity in the idiotypic repertoire.

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A Humanized Anti-Tumor Necrosis Factor-α Monoclonal Antibody That Acts as a Partial, Competitive Antagonist of the Template Antibody

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ABSTRACT

We have constructed several humanized versions of a monoclonal antibody (MAb78) against human tumor necrosis factor-α (huTNF-α) retaining the complementarity-determining regions (CDR) of the original mouse MAb with or without a variable number of original framework region (FR) residues. All versions, except one, showed a loss of binding affinity and neutralizing potency of at least 10-fold compared to the original mouse MAb or its chimeric equivalent. In some cases, however, the decrease in neutralizing potency was significantly greater than the decrease in binding affinity. Two humanized versions showing the greatest dissociation between these two parameters were studied for their capacity to inhibit the neutralizing activity of chimeric or murine MAb78 when used at concentrations that bound but only partially neutralized huTNF-α. One humanized version (MAb78D) was indeed able to do so, whereas the other (MAb78C) was not found to exert any inhibitory activity at all concentrations tested. The antagonistic effect of MAb78D was concentration dependent and could be overcome by increasing the concentrations of chimeric or murine MAb78. Two different models of MAb78-huTNF-α interaction that may help explain the antagonistic activity of humanized MAb78D are discussed.

INTRODUCTION

THE THERAPEUTIC USEFULNESS of rodent monoclonal antibodies (MAb) in humans is severely limited by their immunogenicity.[1,2] Humanization has emerged in the last few years as a feasible approach to obtain MAb of reduced immunogenicity suitable for repeated administrations to humans. Two genetic engineering techniques have been utilized for this purpose. The first leads to the production of chimeric antibodies comprising rodent variable (V) regions linked to human constant (C) regions.[3,4] The second leads to the production of humanized antibodies where only the complementarity-determining regions (CDR) and, if necessary, a limited number of framework region (FR) residues are of rodent origin, whereas all the rest of the molecule is human.[5] Incorporation of some original FR residues has been shown in several instances to be essential in preserving the correct conformation of the antigen-binding sites thereby avoiding unacceptable losses in biological activity.[6] Antibody humanization has allowed the obtainment of MAb that show greatly reduced immunogenicity and extended half-life *in vivo* compared to chimeric antibodies.[7–9]

Human tumor necrosis factor-α (huTNF-α) is a clinically relevant target against which it would be desirable to have available MAb of reduced immunogenicity. It is an inflammatory cytokine and as such plays a pivotal role in host defense against pathogens.[10–13] However, in case of hyperproduction, huTNF-α may lead to harmful consequences and contribute to the pathogenesis of many disease states.[14–17] These observa-

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tions have suggested that MAb against huTNF-α could be therapeutically and/or prophylactically useful. Indeed, animal studies have confirmed that MAb-based prophylaxis and/or therapy of disease states linked to TNF-α hyperproduction is feasible and promising.[18,19] The therapeutic use of currently available rodent MAb in humans, however, will be restricted to those clinical situations that can benefit from a single or few administrations over a brief period of time. For chronic administration, the availability of antibodies of reduced immunogenicity will be mandatory.

These considerations have led us to humanize a neutralizing mouse MAb against huTNF-α raised in our laboratories (MAb78, IgG_1,k; ref. 20). Several versions containing variable numbers of mouse FR residues were constructed and studied in terms of huTNF-α-binding and neutralization. Unexpectedly, for some versions, we observed a decrease in neutralizing potency that was much greater than the decrease in binding affinity. One of the two humanized antibodies that showed the greatest dissociation between these two parameters was shown to act as a partial, competitive antagonist of the original mouse MAb or of its chimeric equivalent.

MATERIALS AND METHODS

Cloning of murine Ig V region DNA

The murine hybridoma 78 secretes an IgG_1,k MAb that neutralizes huTNF-α.[20] Cytoplasmic RNA was prepared from hybridoma cells as described.[21] Ig V region cDNAs were made from RNA *via* reverse transcription initiated from primers based on sequences at the 5′ ends of murine IgG_1 and k C regions[22] as previously described.[6] For VH the primer was CG1FOR (5′-GGAAGCTTAGACAGATGGGGGTGTCGTTTTG) and for the VK the primer was CK2FOR (5′-GGAAGCTTGAAGATGGATACAGTTGGTGCAGC) and contained restriction sites (underlined) for directional cloning. Ig VH and VK cDNA were amplified by polymerase chain reaction (PCR)[23] using a battery of sense primers based on signal and/or amino-terminal V region DNA sequences in concert with the antisense oligodeoxyribonucleotides used to prime the cDNA reactions. Authentic VH sequences were obtained using VH1 BACK[23] and CG1FOR and VK sequences were obtained using VK7BACK (5′-TTGAATTCGGAGCTGATGGGAACATTGTAATG) and CK2FOR. PCR products were gel purified, digested with the appropriate restriction enzymes, and cloned into M13 mp18 and mp19, and the sequences of both strands were determined from at least 12 clones by the chain-termination method.[24] The sequence data of the MAb78 V regions have been submitted to the EMBL/GenBank Databases under accession numbers Z22669 (H chain) and Z22670 (L chain).

Construction of chimeric antibody genes

To facilitate cloning of V region DNA into expression vectors, restriction sites were placed in close proximity to the amino- and carboxy-termini of murine VH and VK genes. For VH, a 5′ *Pst* I and a 3′ *Bst* EII site were introduced into a cloned murine VH region by PCR using VH1BACK and VH1FOR.[23] For VK, a 5′ *Pvu* II and a 3′ *Bgl* II site were introduced into a cloned murine VK region by PCR using VK1BACK and VK1FOR.[23] In some instances, these primers changed one or more amino acids from those naturally occurring (see Fig. 1). The V region genes (MuVH and MuVK) were cut with the appropriate restriction enzymes and cloned into *Pst* I-*Bst* EII-cut M13VHPCR1 and *Pvu* II–*Bcl* I-cut M13VKPCR1[23] which contain an Ig promoter, signal sequences, and splice sites. These DNAs were then excised from M13 as *Hind* III–*Bam* HI fragments and cloned into the expression vectors pSVgpt and pSVhyg[23] containing human IgG_1[25] and human k[26] C regions.

Construction of humanized antibody genes

The starting DNAs were the murine VH and VK genes cloned into M13. Oligonucleotides were synthesized to encode the HIL and LAY FR and the humanized V regions assembled by a series of sequential PCR. M13 clones containing the final humanized V regions were sequenced in their entirety to ensure the absence of spurious mutations, digested with *Pst* I–*Bst* EII or *Pvu* II–*Bgl* II and sequentially cloned into M13VHPCR1 or M13VKPCR1 and then into pSVgpt or pSVhyg as described above. Human IgG_1, IgG_4[27], or k C regions were added as appropriate. Additional humanized constructs with FR mutations were made by overlap/extension and PCR.[28]

Expression of recombinant antibody genes

Approximately 2 μg of the H and 5 μg of the L-chain expression vectors were digested with *Pvu* I and cotransfected by electroporation into YB2/O rat myeloma cells as previously described.[6] gpt^+ transfectants were selected, screened by ELISA for the secretion of human IgG,[6] and expanded.

Quantitation of MAb78 and its humanized versions

Antibody concentrations in supernatants were determined in a quantitative ELISA; those in purified preparations by absorbance at 280 nm using a molar extinction coefficient of 1.4.

Purification of the antibodies

Antibodies were purified by affinity chromatography on Protein G–Sepharose using a commercially available kit (MAb Trap ™G-Kit, Pharmacia, Uppsala, Sweden) and following the manufacturer's instructions. The purity of the preparations thus obtained was >95% as judged by SDS-PAGE.

Affinity measurements

These were performed essentially as described elsewhere.[20] Briefly, graded concentrations of ^{125}I-labeled huTNF-α (NEN, Wilmington, DE) were incubated for 4 hr at room temperature in the absence or presence of MAb78 or of one of its humanized versions. Then, 100 μl of 1:5 diluted Immunobeads (Bio-Rad, Segrate, Italy, anti-mouse Ig for mouse MAb78 and anti-human Ig for humanized MAb) were added to the 400-μl mixtures, and incubation was continued for another hour. Mixtures were then centrifuged through a phthalate-dibutyl phthalate oil mixture

```
A                                       20                  30              40
Murine VH (mAb78)   X V X L Q X S G P G L V Q P S Q S L S I T C T V S G F S L T|G Y G V H|W V R Q S
mAb78A and B VH     Q - Q - - Q - - - - - - - - - - - - - - - - - - - - - - - -|- - - - -|- - - - -
mAb78 C             Q - Q - - Q A - G - V - - - G R - - R L S - I A - - - T F S|- - - - -|- - - - A
mAb78 D             Q - Q - - Q A - G - V - - - G R - - R L S - I A - - - - - -|- - - - -|- - - - A
mAb78 E             Q - Q - - Q A - G - V - - - G R - - R L S - I A - - - - - -|- - - - -|- - - - A
mAb78 F             Q - Q - - Q A - G - V - - - G R - - R L S - I A - - - - - -|- - - - -|- - - - A
mAb78 G             Q - Q - - Q A - G - V - - - G R - - R L S - I A - - - - - -|- - - - -|- - - - A
mAb78 H             Q - Q - - Q A - G - V - - - G R - - R L S - I A - - - - - -|- - - - -|- - - - A

                                      50                  60                  70                  80
Murine VH (mAb78)   P G R G L E W L G|V I W R G G S T D Y N A A F M S|R L R I T K D N S K S Q V F F
mAb78A and B VH     - - - - - - - - -|- - - - - - - - - - - - - - - -|- - - - - - - - - - - - - - -
mAb78 C             - - K - - - - V A|- - - - - - - - - - - - - - - -|- F T - S R - - - - R T L Y M
mAb78 D             - - K - - - - V A|- - - - - - - - - - - - - - - -|- F T - S R - - - - R T L Y M
mAb78 E             - - K - - - - - -|- - - - - - - - - - - - - - - -|- F T - S R - - - - R T L Y M
mAb78 F             - - K - - - - - -|- - - - - - - - - - - - - - - -|- F T - S R - - - - R T L Y M
mAb78 G             - - K - - - - - -|- - - - - - - - - - - - - - - -|- - - - - - - - - - R T L Y M
mAb78 H             - - K - - - - - -|- - - - - - - - - - - - - - - -|- - - - - - - - - - R T - Y -

                                        90                  100                     110
Murine VH (mAb78)   K L N S L R A D D T A I Y Y C A K|N L G I P T F L Y S L D Y|W G Q G T S V T V S S
mAb78A and B VH     - - - - - - - - - - - - - - - - -|- - - - - - - - - - - - -|- - - - - - - - - - -
mAb78 C             E M - - - - T E - - - V - - - - -|- - - - - - - - - - - - -|- - - - V L - - - - -
mAb78 D             E M - - - - T E - - - V - - - - -|- - - - - - - - - - - - -|- - - - V L - - - - -
mAb78 E             E M - - - - T E - - - V - - - - -|- - - - - - - - - - - - -|- - - - V L - - - - -
mAb78 F             E M - - - - T E - - - V - - - - -|- - - - - - - - - - - - -|- - - - - L - - - - -
mAb78 G             E M - - - - T E - - - V - - - - -|- - - - - - - - - - - - -|- - - - V L - - - - -
mAb78 H             E - - - - - T E - - - V - - - - -|- - - - - - - - - - - - -|- - - - V L - - - - -

B                                 10                  20                  30                  40
Murine VH (mAb78)   N I V M T Q S P K S M S M S V G E R V T L S C|K A S E N V D T F V S|W Y Q Q K P
mAb78 A             D - Q L - - - - - - - - - - - - - - - - - - -|- - - - - - - - - - -|- - - - - -
mAb78 B-H           D - Q L - - - - - S - L - V - - - D - - - I T -|- - - - - - - - - - -|- - - - - -

                                    50                  60                  70                  80
Murine VH (mAb78)   E Q S P K L L I Y|G A S N Y R T|G V P D R F T G S G S A T D F T L T I S S V Q A
mAb78 A             - - - - - - - - -|- - - - - - -|- - - - - - - - - - - - - - - - - - - - - - - -
mAb78 B-H           G L A - - - - - -|- - - - - - -|- - - S - - S - - - - G - - - - F - - - - L - P

                                    90                  100
Murine VH (mAb78)   E D L A D Y H C|G Q S Y I F P P T|L G G G T K L E I K
mAb78 A             - - - - - - - -|- - - - - - - - -|- - - - - - - - - -
mAb78 B-H           - - I - T - Y -|- - - - - - - - -|F - Q - - - V - - -
```

FIG. 1. Amino acid sequences of VH (A) and VK (B) from the murine, chimeric, and humanized antibodies. CDR are boxed. Hyphens denote residues that are identical to the corresponding murine V regions. Underlined residues are dictated by PCR primers. Numbering is according to Kabat *et al.*[22]

(1:1.5 v/v) and Immunobead-associated radioactivity was measured. Binding data were then processed, assuming ^{125}I-labeled huTNF-α was a trimer, by means of an equilibrium binding data analysis program (EBDA/HOT, Elsevier Science Publishers, Amsterdam, Netherlands; ref. 29).

HuTNF-α neutralization assays

HuTNF-α (Esquire, Zürich, Switzerland), at a concentration known to exert submaximal cytotoxic activity (1 ng/ml; >85% cytotoxicity), was incubated for 2 hr at 37°C in the absence or presence of graded concentrations of mouse MAb78 or its humanized versions. Mixtures and actinomycin D (2 μg/ml final concentration; Fluka, Buchs, Switzerland) were then added to mouse LM cells. After overnight incubation at 37°C, 5% CO_2, cells were stained with MTT (850 μg/ml final concentration; Prodotti Gianni, Milan, Italy) for 4 hr and optical denisty was then read at 570 nm. Concentrations of MAb78 or its humanized versions neutralizing 50% of the cytotoxic activity of huTNF-α were calculated. The same assay was used to detect a possible

antagonistic effect of humanized MAb78C and D. For this purpose mixtures of huTNF-α with or without mouse or chimeric MAb78 and with or without humanized MAb78C, MAb78D, or an unrelated, isotype-matched (IgG_1,k) humanized MAb were set up and then processed as described before.

RESULTS

V region genes

The V region cDNAs from the murine hybridoma 78 were cloned by PCR using C region oligonucleotides together with primers based on sequences of known signal and/or 5′ sequences of mature V regions. PCR products of the expected sizes for VH and VK were obtained using VH1BACK/CG1FOR and VK7BACK/CK2FOR combinations, respectively. The VH DNA when cloned gave two distinct types. One group consisted of authentic VH sequence but had a deletion encompassing parts of FR3 and CDR3 resulting in a frameshift. We have isolated this nonproductive sequence from other hybridomas in our laboratory and presume its origin is from the NSO fusion partner used in the production of the hybridoma 78. The second VH sequence obtained had an open reading frame and is the putative 78 VH DNA. Only one type of light chain V region sequence was isolated. The deduced amino acid sequences of VH and VK are shown in Fig. 1.

Recombinant antibodies

To maximize retention of high binding affinity by the humanized antibody, we chose to use human FR with highest homology to those of MAb78. The MAb78 VH and VK amino acid sequences were searched against the NBFR-PIR data base. The most homologous mature human VH and VK were, respectively, from the human myeloma protein HIL[29] and from the human IgM antibody LAY.[30] The humanized V regions were constructed by framework mutagenesis of the murine VH and VK genes. Additional alterations to the HIL FR were a Val → Gln change at position 5 to accommodate a *Pst* I restriction site and an Arg → Lys change at position 94, a site known to be critical for retention of affinity.[6] Similarly, to accommodate *Pvu* II and *Bgl* II restriction sites in the humanized VK LAY, residues Met[4] and Val[106] were changed to Leu and Ile, respectively. We made additional versions of the humanized antibody (MAb78C-H) (Table 1) with progressive replacements of particular HIL VH FR amino acids with those from the murine progenitor VH. FR residues that were changed were those that may directly interact with antigen or alter the conformation of the loops. For instance, residues 28 and 30 are structurally a part of the loop incorporating CDR1 and their side chains are surface exposed and may participate directly in antigen interaction.[31] The side chains of residues 48, 49, 67, 78, 80, 82, and 107 are all buried and the internal packing of these residues close to, or below, the CDR loops may indirectly modulate their conformation.[33] Residues 68 and 70 were also changed in some versions so that the entire β-strand proximal to CDR2 was of murine origin.

Binding affinity and neutralizing activity of MAb78 and its humanized versions

Table 1 and Fig. 1 list the isotype and the FR of the MAbs that were studied.

Determination of the affinity constants (Table 2) yielded values that allowed the humanized versions of MAb78 to be divided into two groups. The first, comprising chimeric MAb78A, hybrid MAb78B (comprising mouse H chain V regions and L chain V regions bearing mouse CDRs and fully human FRs), and humanized MAb78H, bearing the highest number of original FR residues of all humanized versions of MAb78, bound ^{125}I-labeled huTNF-α with apparent affinity constants (K_{app}) similar to that of the original MAb78. On the other hand, all other humanized versions of MAb78, including MAb78C that bears minimal FR modifications, bound ^{125}I-labeled huTNF-α with a K_{app} that was approximately one order of magnitude lower than that of MAb78 and of the aforementioned humanized versions. For MAb78C and D, this decrease was independently confirmed in an ELISA measuring binding to solid-phase-bound huTNFα (data not shown).

The neutralizing potency of the same antibodies was deter-

TABLE 1. ISOTYPE AND FRAMEWORK REGIONS OF THE MONOCLONAL ANTIBODIES

MAb (abbreviation)	*Residues changed in human framework regions*[a]		
	Isotype	*H chain*	*L chain*
MAb78	mu IgG_1,k	All	All
MAb78 A	hu IgG_1,k	All	All
MAb78 B	hu IgG_1,k	All	None
MAb78 C	hu IgG_1,k	R94K[b]	None
MAb78 D	hu IgG_1,K	T28S,F29L,S30T,R94K	None
MAb78 E	hu IgG_1,k	T28S,F29L,S30T,V48L,A49G,R94K	None
MAb78 F	hu IgG_1,k	T28S,F29L,S30T,V48L,A49G,R94K,V107T	None
MAb78 G	hu IgG_4,k	T28S,F29L,S30T,V48L,A49G,F67L,T68R,S70T,R71K,R94K	None
MAb78 H	hu IgG_4,k	T28S,F29L,S30T,V48L,A49G,F67L,T68R,S70T,R71K,L78V,M80F,M82L,R94K	None

[a]The heavy-chain framework region sequences are those of the human antibody HIL; the light-chain framework region sequences are those of the human antibody LAY.

[b]Wild-type residue followed by residue number and then mutant residue.

TABLE 2. AFFINITY CONSTANTS (K_{app}) FOR THE BINDING OF ^{125}I-LABELED huTNF-α TO MOUSE MAb78, CHIMERIC MAb78 OR HUMANIZED VERSIONS OF MAb78

	K_{app}	
MAb78	*Exp. 1*	*Exp. 2*
Mouse MAb78	2.44×10^{-11} *M*	4.31×10^{-11} M
MAb78A	2.72×10^{-11} *M*	3.07×10^{-11} M
MAb78B	4.87×10^{-11} *M*	ND[a]
MAb78C	2.3×10^{-10} *M*	3.28×10^{-10} M
MAb78D	3.45×10^{-10} *M*	2.79×10^{-10} M
MAb78E	3.28×10^{-10} *M*	ND
MAb78F	5.49×10^{-10} *M*	ND
MAb78G	4×10^{-10} *M*	2.02×10^{-10} M
MAb78H	2.62×10^{-11} *M*	3.4×10^{-11} M

[a]ND, Not done.

mined by measuring the antibody concentrations required to neutralize 50% of the cytotoxic activity of a huTNF-α dose (1 ng/ml) that exerts a submaximal cytotoxic effect. The results obtained in these experiments are shown in Fig. 2. As can be seen, the pattern of neutralizing potencies relative to MAb78 changed considerably when compared to that of the binding affinities. Here, only the chimeric MAb78A and the hybrid MAb78B neutralized huTNF-α to a degree comparable to that of mouse MAb78. The humanized versions MAb78C and D, bearing one and four mouse FR residues, respectively, had a neutralizing potency ~300-fold lower than MAb78. The versions bearing an increasing number of MAb78 residues in the FR progressively recovered neutralizing potency up to a level that approached the decrease in binding affinity (MAb78G). MAb78H, which had been shown to bind ^{125}I-labeled huTNF-α with an affinity similar to mouse MAb78, did not show a full recovery of neutralizing activity.

Antagonistic activity of MAb78D on the neutralizing activity of chimeric or mouse MAb78

The dissociation between decrease in binding affinity (~10-fold) and neutralizing potency (~300-fold) of MAb78C and MAb78D suggested that at certain concentrations these versions would bind to but not, or only partially, neutralize huTNF-α. In so doing, they might prevent added mouse MAb78 or chimeric MAb78A from binding to huTNF-α and thus antagonize their neutralizing activity. The experiments in Fig. 3 show that in one case this reasoning proved to be correct. Humanized MAb78D inhibited the neutralizing activity of different concentrations of chimeric mAb78A (Fig. 3A). The inhibitory activity of MAb78D was gradually overcome at increasing doses of chimeric MAb78A.

Figure 3B shows the results obtained with MAb78C, the humanized version bearing only one substitution in the FR. In this case, no inhibition of the neutralizing activity of chimeric MAb78A was observed, neither at the concentration indicated in Fig. 3 (900 ng/ml) nor at any of a whole range of concentrations that were tested subsequently (data not shown). Figure 4 shows that MAb78D was able to antagonize mouse MAb78 as well and, on the other hand, that an unrelated, humanized IgG_1k MAb was unable to do so, thereby proving the specificity of the observed inhibition. In a final experiment, shown in Fig. 5, we measured the neutralizing activity of different concentrations of chimeric MAb78A on 1 ng/ml huTNF-α in the absence or presence of different concentrations of humanized MAb78D. As can be seen, increasing doses of MAb78D caused parallel shifts of the dose-response curve of chimeric MAb78A, thus demonstrating that MAb78D inhibited chimeric MAb78A acting as a partial, competitive antagonist.

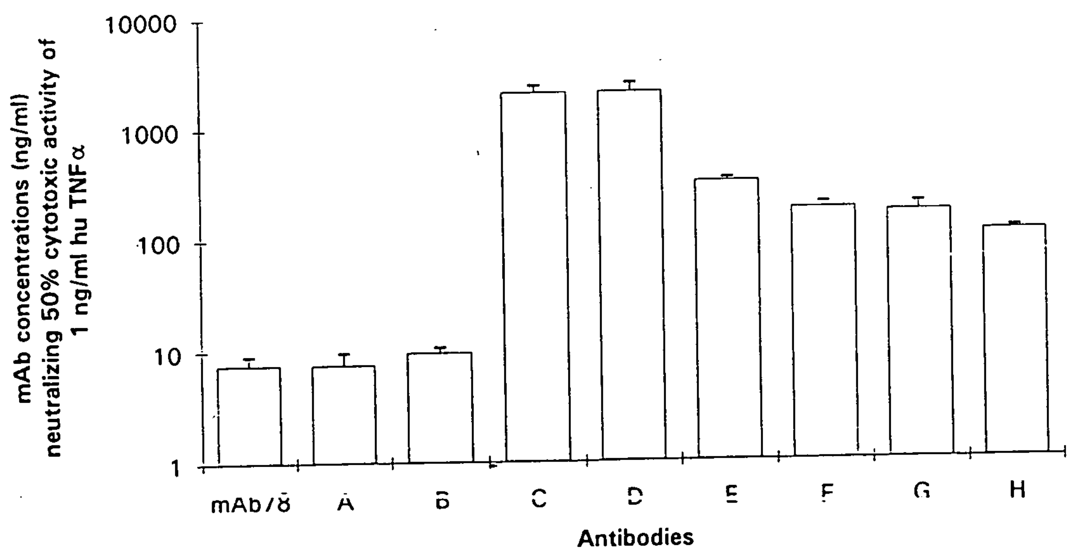

FIG. 2. Concentrations of MAb78 and humanized versions of MAb78 that neutralize 50% of the cytotoxic activity of 1 ng/ml huTNF-α on LM cells. Antibodies were mouse MAb78. (A). Chimeric MAb78A. (B). Hybrid MAb78B. (C). Humanized MAb78C. (D). Humanized MAb78D. (E). Humanized MAb78E. (F). Humanized MAb78F. (G). Humanized MAb78G. (H). Humanized MAb78H.

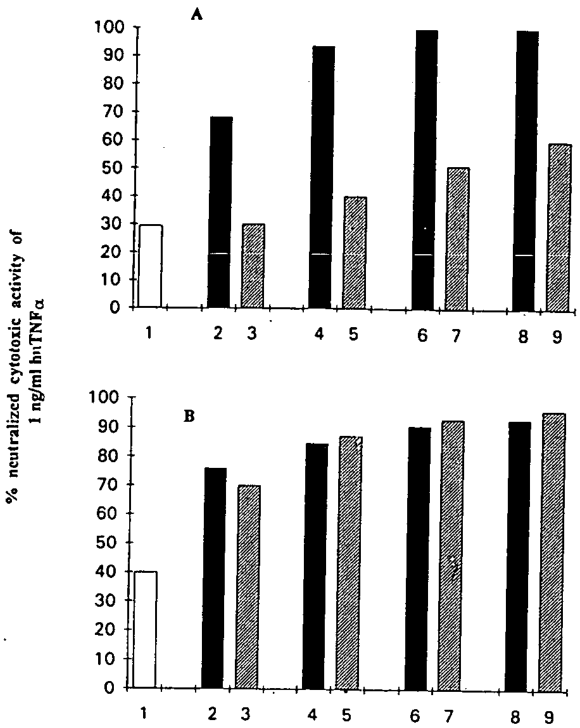

FIG. 3. Cytotoxic activity of 1 ng/ml huTNF-α neutralized by chimeric MAb78A in the absence or presence of humanized versions of MAb78. A was with humanized MAb78D (1,200 ng/ml), B with humanized MAb78C (900 ng/ml). 1, Humanized MAb78 alone; 2, 12.5 ng/ml chimeric MAb78A alone; 3, 1+2; 4, 25 ng/ml MAb78A alone; 5, 1+4; 6, 50 ng/ml MAb78A alone; 7, 1+6; 8, 100 ng/ml MAb78A alone; 9, 1+8.

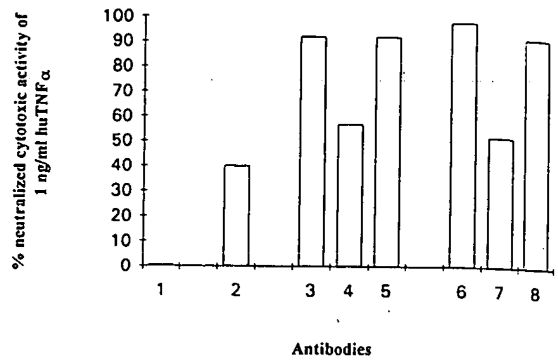

FIG. 4. Cytotoxic activity of 1 ng/ml huTNF-α neutralized by mouse MAb78A in the absence or presence of humanized MAb78D and by mouse or chimeric MAb78D and by mouse or chimeric MAb78A in the absence or presence of an irrelevant, humanized IgG_1,k MAb. 1, 1.8 μg/ml irrelevant, reshaped IgG_1,k MAb alone; 2, 1.2 μg/ml humanized MAb78D alone; 3, 25 ng/ml chimeric MAb78A; 4, 3+2; 5, 3+1; 6, 25 ng/ml mouse MAb78 alone; 7, 6+2; 8, 6+1.

DISCUSSION

We have humanized a mouse MAb against huTNF-α, an inflammatory cytokine involved in the pathogenesis of a large number of disease states.[14–17] Several humanized versions were constructed, using FR with highest homology to MAb78, and tested in terms of binding affinity and neutralizing potency.

A chimeric version bearing the V regions of the original mouse MAb78 and human C regions was equipotent to MAb78 for both parameters studied. Also, a version bearing the mouse heavy-chain V regions, a humanized light-chain V region (mouse CDR and human FR), and human C regions showed no significant decrease in affinity or neutralizing activity. Thus, humanization of the light chain was without any major consequence on the activity of the antibody. The picture changed when we studied humanized versions of MAb78 in which the heavy chain was also humanized. Two versions, one bearing a single original residue in the FR (Lys^{94}) and the other bearing 4 (Lys^{94}, Ser^{28}, Leu^{29}, Thr^{30}), behaved in a similar way. A 10-fold decrease in binding affinity, but a much higher decrease in neutralizing activity (~300-fold) was observed. Upon incorporation of an increasing number of mouse FR residues (MAb78E-G) a neutralizing potency approaching that expected from the decrease in binding activity was recovered progressively. MAb78H, bearing the highest number of original FR residues of all humanized versions, had a binding affinity similar to that of chimeric or mouse MAb78, but the neutralizing activity was still significantly lower than that of MAb78 versions bearing fully murine H chain V regions. Altogether the results obtained with the different humanized versions of MAb78 clearly show that binding affinity and neutralizing potency are dissociable compared to the original, template MAb. The versions containing minimal FR modifications showed the highest degree of dissociation. A similar phenomenon has been reported recently for some humanized versions of an anti-$p185^{HER2}$ MAb.[34]

On the basis of these results, we went on to test if concentrations at which MAb78C and D bound, but did not, or only partially neutralized huTNF-α, were able to antagonize the neutralizing activity of chimeric or mouse MAb78. The experi-

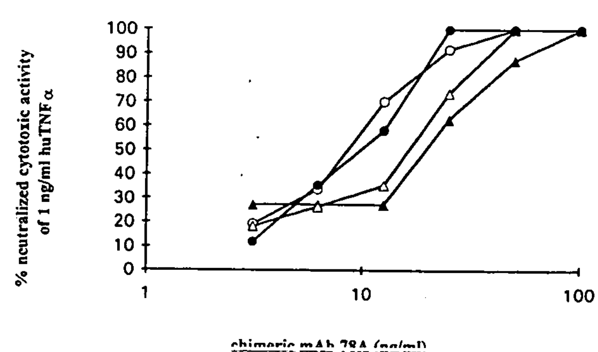

FIG. 5. Cytotoxic activity of 1 ng/ml huTNF-α neutralized by different concentrations of chimeric MAb78A in the absence or presence of different concentrations of humanized MAb78D. (○) chimeric MAb78 alone; ( ) + 200 ng/ml humanized MAb78D; (△) + 400 ng/ml humanized MAb78D; (▲) + 600 ng/ml MAb78D.

ments reported in Figs. 3–5 showed this to be indeed the case for MAb78D. The parallel shifts caused by MAb78D on the dose–response curve of 1 ng/ml huTNF-α in the presence of increasing doses of chimeric MAb78A (Fig. 5) are suggestive of competitive antagonism. This observation is not unprecedented because it has been recently reported that a humanized Fab fragment of an anti-p185^{HER2} MAb inhibits the antiproliferative activity of the chimeric equivalent.[35] At variance with MAb78D, however, MAb78C did not inhibit the neutralizing activity of chimeric MAb78A at all concentrations tested. This is surprising because MAb78C had binding and neutralizing activities very similar to those of MAb78D.

On the basis of the above results, we speculated as to possible modes of huTNF-α–MAb78 interactions that could explain the observed antagonistic activity of MAb78D. A first possibility is that MAb78 leads, upon binding, to a modification of the huTNF-α conformation such that interaction with complementary cell-surface receptors is inhibited. Such a mechanism is referred to as an allosteric effect[36] and has been suggested to operate in several antigen–antibody systems.[37] Thus, compared to murine or chimeric MAb78, humanized MAb78D would bind huTNF-α with an efficiency corresponding to the decrease in affinity but would be far less efficient in causing conformational modifications of huTNF-α. Therefore, at certain concentrations, MAb78D would successfully compete with murine or chimeric MAb78 for huTNF-α binding, thereby leading to an inhibition of the neutralizing activity of the latter antibodies while being by itself unable to neutralize huTNF-α.

A second model can be derived from the observation that huTNF-α, a noncovalently-linked trimer,[38,39] induces the biological response through multisite binding to and subsequent clustering of cell-surface receptors.[40,41] Possibly, MAb78 (murine or chimeric) prevents receptor clustering, thereby leading to neutralization, through binding of a single paratope to huTNF-α. MAb78D would be unable to do so because critically involved FR residues are lacking. Binding of at least a second, perhaps a third paratope and, consequently, higher antibody concentrations, would be required to achieve the same effect. This would explain the difference in neutralizing potency. On the other hand, binding of one MAb78D paratope to huTNF-α would be sufficient to reduce, for steric reasons, the affinity of MAb78 (murine or chimeric) paratope binding to huTNF-α. Therefore, at certain concentrations, MAb78D would bind to huTNF-α without neutralizing it, but at the same time reduce the affinity of binding of murine or chimeric MAb78, thereby causing antagonism.

Both models, however, do not explain why MAb78C was devoid of antagonistic activity. Possibly, determination of the affinity alone, as has been done in the present work, may not be sufficient to characterize the mechanism of binding in all cases. Determination of kinetic and/or thermodynamic parameters[33,35] may be more useful in this regard and, possibly, explain the differential capacity of MAb78C and D to antagonize murine or chimeric MAb78.

In conclusion, we have described in the present report a humanized anti-huTNF-α antibody that acts as a partial, competitive antagonist of the original, template antibody. The results point, at least in the present antigen–antibody system, to a critical role of FR residues in shaping the biological (neutralizing) activity of a MAb. Changing the original FR sequence to a related, but different one, converted a neutralizing antibody into one with greatly reduced activity, while the loss in antigen-binding activity was much less important.

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Brief Definitive Report

MODULATION OF ENDOTHELIAL CELL HEMOSTATIC PROPERTIES BY TUMOR NECROSIS FACTOR

BY PETER P. NAWROTH AND DAVID M. STERN

From the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Recent studies (1, 2) have indicated that endothelium provides a focal point for the interaction of mechanisms inhibiting and promoting activation of coagulation. Perturbation of endothelium can induce procoagulant activity, allowing these cells to initiate and propagate an entire coagulation pathway leading to the deposition of fibrin (2). Thus, modulation of endothelial cell coagulant properties could play a role, comparable to the traditionally accepted leukocyte procoagulants, in promoting fibrin deposition in response to inflammatory and neoplastic stimuli.

Prompted by this hypothesis and the recently described (3) identity between tumor necrosis factor (TNF) and cachectin, a mediator of the host response in gram-negative sepsis (4), we have examined the effect of TNF on the coagulant properties of endothelium. The results indicate that cultured bovine aortic endothelial cells incubated with recombinant TNF have enhanced procoagulant activity, tissue factor, and concomitant suppression of the protein C pathway, an antithrombotic mechanism that functions on the surface of quiescent endothelial cells. This unidirectional shift in endothelial cell hemostatic properties promoting clot formation provides insight into the intercellular signaling by which a monokine generated in response to sepsis can modulate the role of endothelium in coagulation.

Materials and Methods

Cell Culture. Bovine aortic and human umbilical vein endothelial cells were cultured and characterized as previously described (5).

Treatment of Endothelial Cells with TNF. Experiments were carried out 24 h after endothelial cells reached confluence using primary cultures and serially passaged cells (passages 1–8). Monolayers were washed three times with HBSS (Gibco Laboratories, Grand Island, NY), serum-free medium was added along with TNF, and cells were incubated for the indicated times at 37°C. After the incubation period, cultures were washed three times with incubation buffer and assayed for tissue factor activity (9.6-cm^2 wells), the ability to promote thrombin-mediated protein C activation (0.32-cm^2 wells), or the ability to promote activated protein C–protein S–mediated Factor Va inactivation (0.79-cm^2 wells).

Reagents and Assays. Recombinant human TNF, generously provided by BASF (Ludwigshafen, Federal Republic of Germany), was used for all studies. This TNF preparation, which was free of detectable endotoxin at the concentrations used in these experiments, migrated as a single band with an M_r of 17,000 under both reducing and nonreducing conditions on SDS-PAGE. This material has identical properties to previously described

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(6) preparations of TNF (a manuscript concerning the detailed characterization of this TNF is in preparation by BASF). Heat treatment of TNF was carried out at 70°C for 1 h, and resulted in loss of cytolytic activity and loss of its effect on endothelial cell coagulant parameters (Fig. 1).

All coagulation factors were of bovine origin. The synthetic substrate tissue factor assay (Table I) (7) was carried out by adding purified Factors VIIa (8 nM) (generously pr vided by Dr. W. Kisiel, University of New Mexico, Albuquerque, NM) and X (1.3 μM) (2) to monolayers in incubation buffer (1 ml) for 10 min at 21°C. Where indicated, rabbit anti-tissue factor IgG was added (200 μg/ml) (8) (generously provided by Dr. R. Bach, Mt. Sinai School of Medicine, New York) for 30 min before the assay. One sample (0.1 ml) was removed from each reaction mixture and assayed for Factor Xa by monitoring (at 405 nm) hydrolysis of the chromogenic substrate benzo-Ile-Glu-Gly-Arg-*p*-nitroanilide (Helena Labs, Beaumont, TX) (0.1 ml; 0.05 nM) (2). Factor Xa formation was linear, only limited by the amount of tissue factor. Tissue factor activity was also assessed using a one-stage coagulant assay as previously described (7). The amount of tissue factor present was determined by comparison of the clotting time with a standard curve using the same coagulant assay and purified bovine tissue factor inserted into phospholipid vesicles (8) (generously provided by Dr. R. Bach).

Thrombin-mediated protein C activation was studied, as described previously (9), by incubating samples with thrombin (0.1 U/ml) and protein C (85 μg/ml) for 40 min at 37°C in incubation buffer (0.1 ml). Antithrombin III (0.2 mg/ml) was then added to neutralize residual thrombin, the reaction mixture was assayed for activated protein C by monitoring (at 405 nm) hydrolysis of the chromogenic substrate D-Phe-Pip-Arg-*p*-nitroanilide (Helena Labs; 0.05 ml; 2 mM). Activated protein C formation over endothelial cell monolayers was linear over the time of the assay.

Protein S and Factor Va were purified to homogeneity as described (10). Activated protein C–protein S–mediated Factor Va inactivation was studied by adding activated protein C (1 nM), protein S (2 nM) and Factor Va (70 nM) for 1.5 min at 21°C to monolayers in incubation buffer (0.5 ml). Samples were removed, one from each reaction mixture, and assayed for residual Factor Va activity using one-stage clotting assay (10). Rates of Factor Va inactivation were determined from the slope of the linear initial portion of a plot of Factor Va activity versus incubation time.

Results

In contrast to the cytotoxic effect of TNF reported for tumor cells in vitro (3, 6), TNF did not exert its effect on cultured endothelial cells only via direct cytotoxicity. Rather, TNF specifically modulated endothelial cell hemostatic properties. Tissue factor, a cofactor initiating coagulation (8), is not normally expressed on the surface of endothelial cells. Incubation of cultured bovine aortic endothelial cells with TNF led to a dose-dependent induction of tissue factor (Fig. 1*A*). The identity of this procoagulant activity as tissue factor was confirmed by the Factor VIIa–dependence of Factor X activation, which could be prevented by anti–tissue factor IgG (Table I). Although neoplastic tissue has been reported (11) to have a direct Factor X activating enzyme, TNF did not induce significant amounts of this activity in endothelium (Fig. 1*A*, and Table I). Destruction of TNF cytolytic activity by heat treatment also prevented the induction of endothelial cell tissue factor, indicating a requirement for the functionally active molecule (Fig. 1). Similar heat-treatment of endotoxin had no effect on the induction of endothelial cell tissue factor (data not shown). Tissue factor activity induced by TNF was evident after a 2-h lag, increased steadily up to 10 h, and thereafter slowly declined (Fig. 1*B*). This is similar to the decay of IL-1-induced endothelial cell tissue factor activity (12), though th decline appears more

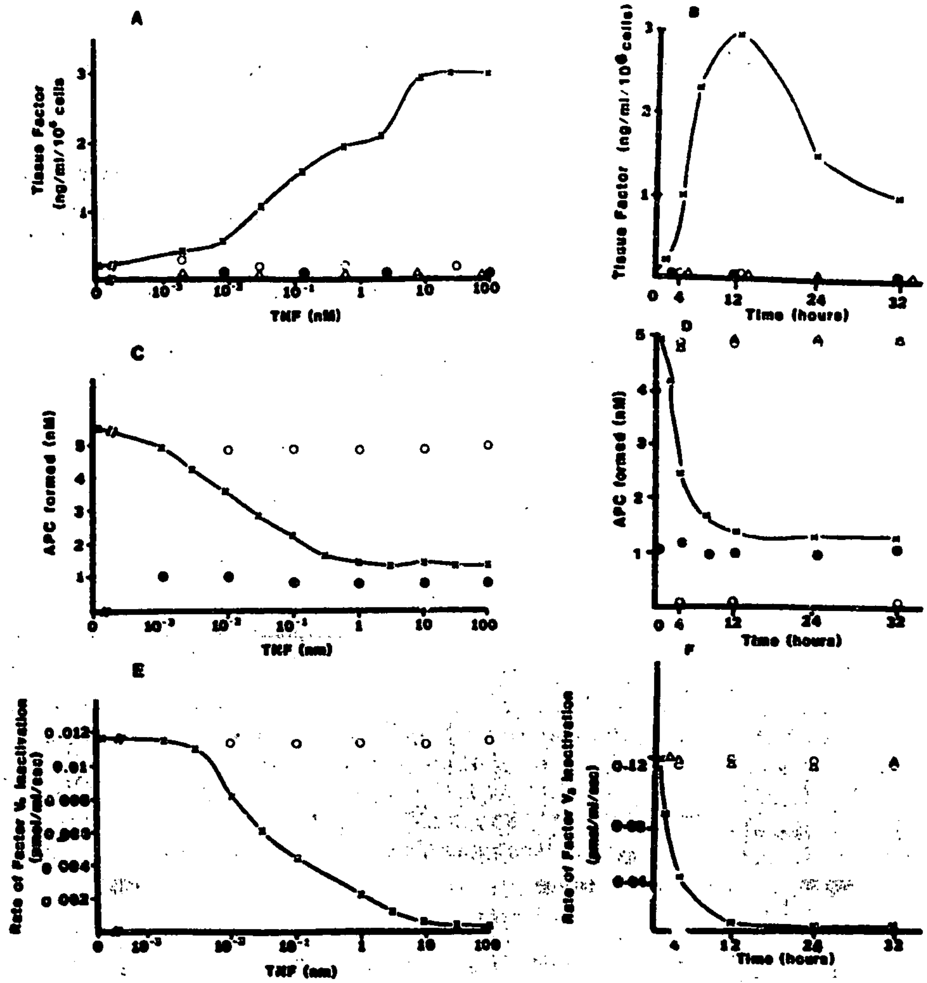

FIGURE 1. Effect of TNF on endothelial cell hemostatic properties. Cultured bovine aortic endothelial cells were incubated with TNF and the effect on endothelial cell tissue factor (*A*, *B*), thrombin-mediated activated protein C formation (*C*, *D*) and activated protein C–protein S–mediated Factor Va inactivation (*E*, *F*) was assessed. (*A*) Dependence of tissue factor induction on the dose of TNF. Monolayers were incubated with the indicated concentration of TNF (×) for 12 h. Tissue factor activity of endothelium was assessed by a coagulant assay as described in the text. Where indicated, Factor VII–deficient plasma replaced normal plasma (Δ). Heat-treated TNF (○) or cycloheximide (2 μg/ml) (●) were added to certain cultures. (*B*) Time course of tissue factor induction. Monolayers were incubated in serum-free medium alone (Δ) or in the presence of TNF (10 nM) (×). Cycloheximide (●) or heat-treated TNF (○) was added where indicated. Tissue factor was assayed as described in Materials and Methods. Where indicated, Factor VII–deficient plasma was used (▲). (*C*) Dependence of decreased thrombin-mediated protein C activation on the dose of TNF. Monolayers were incubated with the indicated concentration of TNF (×) or heat-treated TNF (○). Endothelium was then incubated with thrombin and protein C, as described in the text, to assess its ability to promote thrombin-mediated protein C activation. Where indicated, goat anti–rabbit thrombomodulin IgG (150 μg/ml) was preincubated with endothelium for 30 min (●). Control IgG from nonimmune animals had no effect on the assay. Results are expressed as activated protein C (*APC*) formed per 40 min per 10^5 cells. (*D*). Time course of decreased thrombin-mediated protein C activation. Monolayers were incubated with TNF (10 nM) (×), heat-inactivated TNF (○), or with serum-free medium alone (Δ) for the indicated times, and washed. Where indicated, goat anti–rabbit thrombomodulin IgG (150 μg/ml) (generously provided by Dr. N. Esmon, Oklahoma Medical Research F undation) was preincubated with endothelium for 30 min (●). Endothelium was th n incubated with thrombin and protein C as described in the text to assess its ability to promote thrombin-mediated protein C activation. Results are xpressed as activated protein C (*APC*) f rmed per 40 min per 10^5 c lls. (*E*) Dependence of decreased

TABLE I

Activation of Factor X by TNF-treated Endothelial Cells

Cell treatment	Assay reaction mixture	Factor Xa formed (pmol/10^6 cells)
None	VIIa, X	3 ± 1
TNF	VIIa, X	108 ± 17
TNF	X	4 ± 1
TNF	VIIa, X, anti-tissue factor IgG	11 ± 1

Endothelial cell monolayers were incubated with or without TNF (10 nM) for 12 h in serum-free medium and washed with incubation buffer. The tissue factor assay was then carried out using Factors VIIa and X as described in the text. Values are expressed as Factor Xa formed within 10 min.

gradual in the case of TNF. Procoagulant activity on the endothelial cell surface was not due to expression from a preformed pool, since lysates of control endothelial cells did not have significant tissue factor activity. Furthermore, cycloheximide (2 μg/ml) blocked the induction of tissue factor by TNF, indicating a requirement for de novo protein synthesis (Fig. 1*B*). Thus, TNF induces endothelium to synthesize and express tissue factor. Similar results were observed with cultured human umbilical vein endothelial cells (data not shown).

In view of the potent anticoagulant mechanisms operative on the endothelial cell surface, induction of procoagulant activity may not be sufficient for the vessel surface to play a role in the pathogenesis of a prethrombotic state. In this context, the importance of endothelial cell participation in the regulation of coagulation is exemplified by the thrombotic diathesis observed (13) in kindreds deficient in protein C or protein S, since function of the protein C anticoagulant pathway is dependent on cofactors present on the endothelial cell surface. Endothelium provides both a cell surface protein, thrombomodulin (25), enhancing thrombin-mediated formation of the anticoagulant enzyme–activated protein C, and cofactor activity promoting assembly of activated protein C–protein S complex, which is the functionally effective anticoagulant unit of the protein C pathway. Activated protein C formation was in large part dependent on the presence of endothelial cell thrombomodulin, as indicated by the 75% inhibition of protein C activation in the presence of antithrombomodulin IgG (Fig. 1*C*). TNF resulted in a dose-dependent decrease in endothelial cell–dependent thrombin-mediated protein C activation. At a TNF concentration of 30 pM, thrombomodulin-dependent protein C activation was decreased by ~50%. Decreased activated protein C formation was also dependent on the

activated protein C–protein S–mediated Factor Va inactivation on the dose of TNF. Monolayers were incubated with the indicated concentration of TNF (×) or heat-treated TNF (○) for 12 h. Endothelium was then assayed for the ability to promote activated protein C–protein S–mediated Factor Va inactivation after incubation with activated protein C, protein S, and Factor Va, as described in the text. Results are expressed as the rate of Factor Va inactivation per 10^5 cells. (*F*) Time course of decreased activated protein C–protein S–mediated Factor Va inactivation. Monolayers were incubated with TNF (10 nM) (×), heat-inactivated TNF (○), or with serum-free medium alone (Δ) for the indicated times, and washed. Endothelium was then assayed for the ability to promote activated protein C–protein S–mediated Factor Va inactivation after the addition of activated protein C, protein S, and Factor Va, as described in the text. Results are expressed as the rate of Factor Va inactivation per 10^5 cells.

incubation time f endothelium with TNF, with an effect evident by 1 h and maximal after 6 h (Fig. 1 *D*). Controls indicated that decreased protein C activati n was due to decreased formation of activated protein C rather than inactivati n f the thrombin added or the activated protein C formed.

Recent studies (10) have shown effective assembly of functional activated protein C–protein S complex on the endothelial cell surface promoting Factor Va inactivation, and thereby regulating thrombin formation. Activated protein C is the enzyme and protein S functions as the cell surface cofactor (10). In contrast to the rapid Factor Va inactivation observed with control monolayers, after incubation with TNF, the rate of Factor Va inactivation was reduced (Fig. 1 *E*). This affect was half-maximal at a TNF concentration of 40 pM. The rate of Factory Va inactivation was attenuated in a time-dependent manner after the addition of TNF to cultures with negligible rates after 8 h (Fig. 1 *F*).

Discussion

The results reported here indicate that TNF induces endothelial cell tissue factor while suppressing endothelial cell–dependent protein C activation and anticoagulant function on the cell surface. Comparison of the time course and dose-response curve for TNF modulation of these distinct cellular coagulant properties indicates striking similarities in all cases (Fig. 1). This suggests that expression of these hemostatic properties may be under coordinate control at the intracellular level. Since a concerted change in the endothelial cell surface promoting the activation of coagulation involves both anticoagulant and procoagulant properties, such a linked control mechanism seems logical. The result of these changes is indicated by previous studies (2) showing that the induction of tissue factor in endothelial cells initiates a procoag lant pathway leading to the deposition of fibrin. Loss of effective function of the protein C anticoagulatnt pathway further enhances the propagation of procoagulant reactions on the endothelial cell surface. The net result of TNF–endothelial cell interaction is a unidirectional shift in the balance of anticoagulant and procoagulant mechanisms on the endothelial cell surface from the quiescent state in which anticoagulant mechanisms predominate, to a stimulated state in which procoagulant activities are dominant.

Fibrin deposition and activation of macrophages around malignant tissues, potentially resulting in local TNF release, is commonly observed by pathologists. Furthermore, the histologic description of the effect of TNF on tumors in vivo is often referred to as hemorrhagic necrosis. This leads to the hypothesis that local effects of TNF on endothelial cell coagulant properties could result in a coagulopathy, interrupting normal blood flow to the tumor and leading to necrosis. This suggests a model in which monokines alter endothelial cell hemostatic properties promoting clot formation, potentially limiting pathologic processes such as infection and tumors.

Summary

Tum r necrosis factor/cachectin (TNF) is a mediator of the septic state, which inv lves diffuse abn rmalities of coagulation thr ughout the vasculature. Since

previous studies have shown that endothelial cells can play an active role in coagulation, we wished to determine whether TNF could modulate endothelial cell hemostatic properties. Incubation of purified recombinant TNF with cultured endothelial cells resulted in a time- and dose-dependent acquisition of tissue factor procoagulant activity. Concomitant with enhanced procoagulant activity, TNF also suppressed endothelial cell cofactor activity for the anticoagulant protein C pathway; both thrombin-mediated protein C activation and formation of functional activated protein C–protein S complex on the cell surface were considerably attenuated. Comparable concentrations of TNF (half-maximal affect at ~50 pM) and incubation times (half-maximal affect by 4 h after addition to cultures) were required for each of these changes in endothelial cell coagulant properties. This unidirectional shift in cell surface hemostatic properties favoring promotion of clot formation indicates that, in addition to leukocyte procoagulants, endothelium can potentially be instrumental in the pathogenesis of the thrombotic state associated with inflammatory and malignant disorders.

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Application Number 08/602,272

1. American Journal of Cardiology, 1991, 68(7):B36-B50
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Thanks!

Identifying Patients at Risk for Thromboembolism

Use of ^{125}I-Labeled Fibrinogen in Patients With Acute Myocardial Infarction

Natalio Cristal, MD; Josef Stern, MD; Menachem Ronen, MD; Carlos Silverman, MD; Winston Ho, MD; Elisha Bartov, MD

Fibrinogen labeled with iodine 125 was used to detect deep vein thromb sis (DVT) in 35 patients during their course and convalescence from acute myocardial infarction. Clinical status was assessed and scored with th use of a modified coronary prognostic index.[1] According to the prognostic scores, patients were allocated to one of two groups. Of 27 patients in good clinical condition, DVT developed in only one patient, whereas thromboembolic complications occurred in seven of eight patients who were sev rely ill—a highly significant difference. Prophylactic anticoagulation is advisable in patients at risk.

(*JAMA* 236:2755-2757, 1976)

IT IS widely accepted that anticoagulants are effective in preventing the thromboembolic complications of acute myocardial infarction (MI).[2,3] Because the presently available anticoagulant drugs are not invariably effective and are not always safe, it seems wise to reserve their use for those patients in whom the risk of developing thromboembolic complications is high. Russek et al[4] in 1953, and more recently, Ebert[5] in 1972, pointed out the importance of defining the patient at risk for the development of thromboembolic complications during acute MI.

The ^{125}I-labeled fibrinogen technique is an effective method for the early detection of deep venous thrombosis (DVT) in the legs.[6-10] It has paved the way for a better understanding of the natural history of thromboembolic complications and has provided a means for defining patients who are at risk of developing them.

The present study was undertaken to evaluate the incidence of DVT in an unselected series of patients with acute MI in an attempt to define the characteristics of the thromboembolism-prone patient.

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METHODS AND PATIENTS

During the period of the study, every patient admitted to the coronary care unit (CCU) received potassium iodide, 120 mg orally, on the day f admission. Patients in whom the diagnosis of acute MI was confirmed by development of Q waves in the ECG or by a substantial rise in the levels of serum SGOT and creatine phosphokinase were selected for the study. They received 1 mg of ^{125}I-labeled fibrinogen (containing 100μCi) intravenously 24 hours after admission. In order to block the uptake of the ^{125}I by the thyroid gland, potassium iodide, 60 mg, was given daily for two weeks. Radioactivity in the patients' legs was measured at points marked off every 8 cm along a line between the femoral vein at the junction of the inguinal ligament and the posterior region of the medial malleolus. Measurements were done two and 24 hours after the administration of the ^{125}I-labeled fibrinogen and then every one to two days for the next 10 to 14 days. The patients were examined with use of a nuclear spectrometer, which records scintillation counts by means of a thallium-activated sodium iodide crystal and a photomultiplier unit contained within a glass shield together with a collimator of the 3.75-cm well type.

The scanning was performed while the legs were elevated on a frame at an angle of 25° to the horizontal in order to minimize blood pooling. Counts were made at each position for 30 seconds and were expressed as a percentage of the precordial count recorded at the same session.

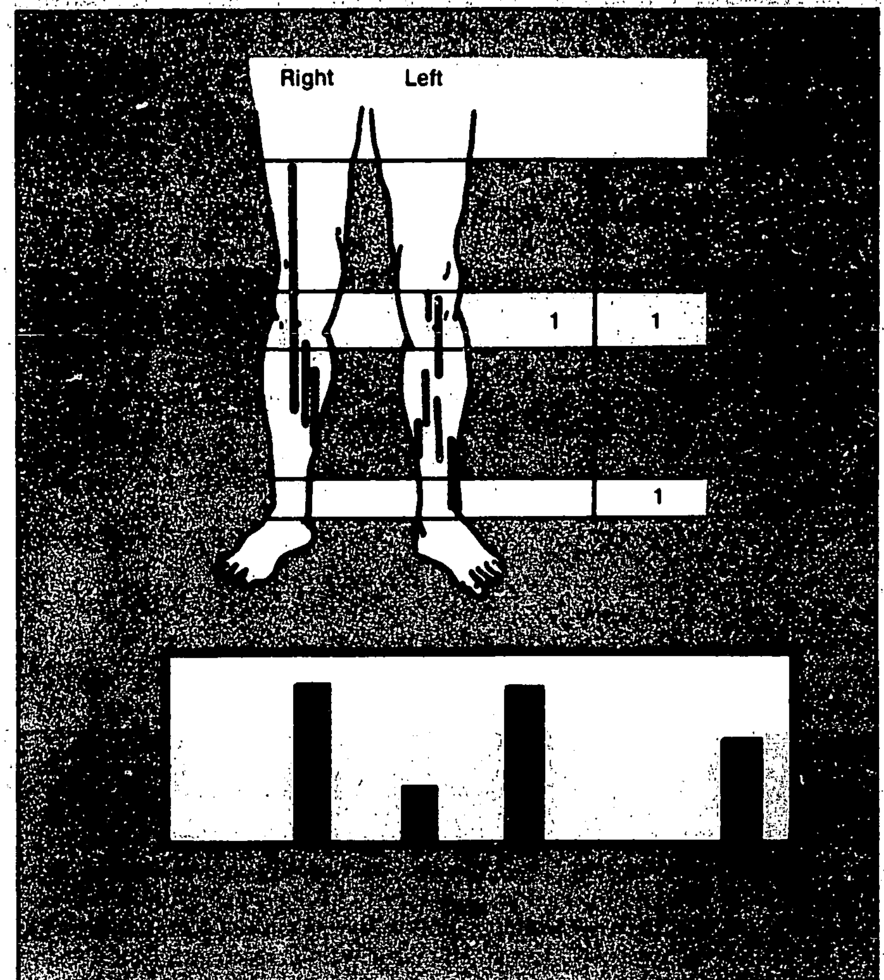
A radioisotopic diagnosis of DVT was made if a difference of 20% or more between corresponding points on the two legs or adjacent points on the same leg lasted for more than 24 hours. A difference f more than 30% in either of those cases was considered DVT even if it was present for less than 24 hours. Daily examinations f r

clinical signs of DVT were performed.

Thirty-five patients were included in the study. Of these, 11 were women and 24 were men. The mean age was 63 years, with a range of 49 to 90 years. In general, the patients were instructed in physiotherapy exercises and were free to m ve in bed. Early ambulation was encouraged, and patients were allowed to sit by the bedside as soon as their status permitted. The mean stay in bed was five days. None of the patients received prophylactic anticoagulant therapy, and only one patient received anticoagulants after the development of pulmonary embolism.

The clinical condition was assessed on admission and was scored according to the coronary prognostic index (CPI) proposed by Norris et al.[1] With each fluctuation in the patient's condition, the CPI was rescored. The reassessment of the CPI was performed without knowledge of the development of isotopical evidence of DVT. It should be noted that only three among the six variables used to construct the Norris CPI may change during the course of the disease (blood pressure, heart size, and lung fields) while the remaining three (age, position of the infarction, and previous ischemia) cannot change in a single admission.

According to his maximal score, every patient was allocated to one of two groups: those with a CPI score of 10 or higher (there were eight patients in this category), and those with a CPI score of less than 10 (there were 27 patients in this category).



Schematic drawing shows location of thrombi and day on which they were diagnosed. Three thrombi were present in right leg, six in left. Graph shows incidence by number of days after myocardial infarction.

RESULTS

Deep vein thrombosis was diagnosed isotopically in six patients (5 men and 1 woman). In two patients, calf pain was noted; in a third, pulmonary embolism was diagnosed on the tenth day. In three patients, DVT appeared only in the left leg; in another three, the isotopical evidence of DVT appeared in both legs. One thrombus was confined to the left popliteal fossa, seven thrombi developed in the calf, and one was located in the ankle zone that extended toward the calf. Among the seven thrombi that developed in the calf, one progressed from the right calf proximally to the thigh. The day on which the thrombi became isotopically evident in these patients is depicted in Fig 1.

Among 27 patients with CPI scores less than 10, only one patient had isotopic evidence of DVT, while five out of eight whose CPI score was 10 points or more had the complication.

It is remarkable that in two of the remaining three patients in the last group, evidence of mural thrombi was found. One patient had an acute occlusion of the left femoral artery; the embolus extracted from the site of occlusion showed evidence of radioactivity. In the second patient, a mural thrombus was found at autopsy three weeks after the acute MI. Since mural thrombi are themselves thromboembolic complications, these findings raise the thromboembolic complication rate in this group to seven of eight.

COMMENT

The use of the ^{125}I-labeled fibrinogen test has shown that in 20% to 40% of patients with acute MI, radioisotopic evidence of DVP develop in the lower limbs.[11-15] These figures are somewhat surprising. Myocardial infarction is a dangerous disease, but in most patients it is not a prostrating one. The majority of patients with MI are, after relief of pain, in a clinical condition not much different from that of patients suffering from diseases in which thromboembolic complications seldom develop. In 1953, Russek[4] pointed out that patients with acute MI could be divided into two groups: one had a very low mortality and also had a low incidence of thromboembolic complications; the other had a high mortality and a high incidence of thromboembolic complications.

This suggestion is supported by our finding that in the group defined in our study as low risk, only one of 27 patients had radioisotopic evidence of DVP, whereas such evidence was present in five of eight patients at high risk (ie, suffering from myocardial damage that resulted in hemodynamic derangement clinically manifested as hypotension, shock, or congestive heart failure). Tw patients of the remaining three in this

Results of Studies Correlating Patient Condition and Occurrence of DVT*						
	No. of Patients	DVT	CPI <10	DVT	CPI >10	DVT
Murray et al[11]	35	12	...	...	...	...
Nicolaides et al[13]	31	6	25	3	6	3
Maurer et al[12]	100	34	77	27	12	7
Kotilainen[14]	52	11	37	4	13	7
Simmons et al[15]	89	24	26	1	63	23
Present study	35	6	27	1	8	5

*DVT indicates deep vein thrombosis; CPI, coronary prognosis index.[1]

category had mural thrombi, making the overall incidence of thromboembolic complications in this group quite high.

The results of similar works are summarized in the Table. Although the definition of "high risk" or "severely ill" patients differs from study to study, the incidence of DVT in each study was substantially higher in the patients at high risk than in those not considered to be severely ill. The incidence of DVT in the patients at low risk ranged from 1 in 26[15] to 27 in 77 patients.[12] The wide range probably reflects differences in the populations studied, in the amount of activity allowed the patient in bed, and in the different criteria used for the radioisotopic diagnosis.

Little progress has been made in our understanding of the mechanisms responsible for the development of thromboembolic phenomena since the three-factor hypothesis of Wirchow et al.[16] Since substantial alterations in the blood components or the venous endothelium have not been described during the acute phase of MI, it seems likely that the third factor, namely, slowing of the blood flow in the venous system, is probably responsible.[16] Conditions causing slowing of the venous blood flow are present in patients with hemodynamic derangements (ie, congestive heart failure, venous ectasis, low cardiac output, anoxia, and poor hydration). Prolonged bed rest with decreased muscular activity is also conducive to slowing of venous flow.

The CPI as proposed by Norris et al in 1969[1] provides an objective method of scoring these complications, provided it is reassessed according to fluctuations in the patient's clinical condition. We chose a score of 10 as the dividing line between the high risk and low risk patients because, in order to reach this score, either hypotension or congestive heart failure had to be in evidence.

The results of our study show that the CPI scoring system is an easy and effective method for evaluating the severity of MI. It offers enough sensitivity and specificity for detection of patients at high risk of developing thromboembolic complications.

We believe that the patients thus defined should receive anticoagulant therapy unless serious contraindications exist.

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24. Throb Haemost, 1987, Vol. 57, pp. 176-182

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Endog nous cytokine antagonists during myocardial ischemia and thrombolytic therapy

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We tested the idea that cytokine antagonists are released during acute myocardial ischemia to counteract proinflammatory effects of cytokines. We investigated changes in plasma concentrations of the anticytokine molecules α–melanocyte-stimulating hormone (α-MSH), interleukin-1 receptor antagonist (IL-1ra), and soluble tumor necrosis factor receptor (sTNFr) in patients with acute myocardial infarction (AMI) or unstable angina (UA). Blood samples were collected at presentation in the coronary care unit, at 3-hour intervals for 24 hours, and daily for 4 days thereafter. There were no significant differences in the concentrations of cytokine antagonists in patients with AMI or UA. However, whereas concentrations of α–MSH were increased in early samples of patients with AMI or UA who were treated with a thrombolytic agent, they were consistently low in untreated patients. IL-1ra concentrations likewise were greater 3 and 6 hours after treatment in patients who underwent thrombolysis, whereas there was no significant difference in plasma sTNFr between the two groups. We suggest that during myocardial ischemia and thrombolysis anticytokine molecules released from the injured myocardium become available to reduce inflammation caused by cytokines and other mediators of inflammation. (Am Heart J 1995;130:204-11.)

Coronary artery inflammation may contribute to the pathogenesis of unstable coronary syndromes, including myocardial infarction.[1] Inflammation may alter the integrity of endothelium and cause exposure of thrombogenic material in the plaque with clot formation and reduction of coronary flow. Myocardial reperfusion after ischemia also causes an inflammatory response with leakage of plasma constituents into the interstitial space and accumulation of polymorphonuclear leukocytes and macrophages at the site of injury.[2] Numerous deleterious substances are released by the invading cells, including superoxide anion, hydrogen peroxide, thromboxanes, and leukotrienes.[2] These substances injure further myocardial cells already damaged by hypoxia. Thus the magnitude of tissue injury depends on degree and duration of the initial ischemic insult and on the effects of toxic products released by infiltrating inflammatory cells. That the inflammatory response contributes to cardiac tissue injury is clear from the protection of reperfused myocardium afforded by depletion of circulating neutrophils.[3,4]

In addition to "classic" mediators of inflammation, activated monocytes and macrophages release proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and interleukin-6 (IL-6).[5] These cytokines cause local inflammation and systemic acute phase responses, including fever, leukocytosis, hepatic protein synthesis, and release of adrenocortical hormones. A proportion of patients with acute myocardial infarction (AMI) have increased plasma concentrations of TNF and IL-6.[6-10] Increased production of IL-6 may account for aspects of the acute-phase response observed in patients with AMI.[8,9]

Cytokine antagonists have been identified, and it is clear that these molecules can protect the host against the detrimental effects of cytokines. Interleukin-1 receptor antagonist (IL-1ra) is an 18 kDa protein that binds to type I and II IL-1 receptors without inducing cell signaling, thereby antagonizing the effects of IL-1 in vivo and in vitro.[11] Effects of TNF are inhibited by soluble forms of its receptors (sTNFr): 55 and 75 kDa TNF receptors, shed from the cell surface, bind the cytokine in the circulation and reduce its effects. Research has shown increases in plasma concentrations of IL-1ra and sTNFr in patients with AMI.[10] The neuropeptide α–melanocyte-stimulating hormone (α-MSH) is an anticytokine

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Table I. Clinical findings in the AMI group

Patient no.	Age (yr)	Sex	AMI site	Time from onset of chest pain (hr)	Thrombolytic therapy	Killip's class	EF* (%)	Complication
1	55	M	Inferior	3	SK	I	60	Silent ischemia
2	68	F	Anterior	4	SK	I	40	
3	68	M	Inferior	2	SK	I	45	Postinfarction angina
4	57	M	Anterior, recurrent	9	SK	I	40	EF reduction
5	64	M	Inferior	13	SK	I	56	
6	65	M	Inferior	5	SK	I	60	
7	64	F	Inferior	3	SK and UK	I	65	Tamponade by heart rupture
8	59	F	Non–Q-wave	3	SK	I	60	
9	25	M	Inferior	3	None	I	60	
10	48	M	Non–Q-wave	6	None	I	50	Silent ischemia
11	63	F	Inferior and posterior	5	SK	I	65	
12	66	M	Anterior	10	rTPA	I	55	
13	38	M	Anterior	3	SK	I	54	Atrial fibrillation
14	51	M	Anterolateral	4	rTPA	I	55	
15	58	M	Inferior, posterior, and lateral	5	SK	I	60	
16	67	M	Anterior	8	SK	I	45	Atrial fibrillation
17	46	M	Anteroseptal	5	SK	I	55	
18	66	M	Inferior and anterolateral, recurrent	4	SK	I	41	
19	53	M	Anterior	1.5	SK	I	70	
20	51	M	Inferior	3	SK	I	65	
21	64	M	Inferior	21	rTPA	I	50	
22	68	F	Lateral	3	SK	I	55	
23	53	M	Inferior	2	rTPA	I	65	
24	69	M	Anterior	3	rTPA	I	55	
25	53	M	Anterolateral	3	rTPA	I	40	
26	54	M	Inferior	2	rTPA	I	60	

EF, Ejection fraction; *SK*, streptokinase; *UK*, urokinase.
*Data compiled from American Society of Echocardiography Committee on Standards. J Am Soc Echocardiogr 1989;2:358-67.

molecule with a broader spectrum of action.[12-14] This 13–amino acid proopiomelanocortin derivative antagonizes proinflammatory effects of IL-1, IL-6, TNF, and other mediators of inflammation, including platelet-activating factor (PAF), leukotriene B_4, and interleukin 8 (IL-8).[15, 16] Although understanding of the mechanism of action of α-MSH is incomplete, its potent antiinflammatory effects have been documented in many models of inflammatory and infectious disorders that involve cytokines.[12-14]

We tested the idea that cytokine antagonists are released into the circulation during myocardial ischemia to counteract proinflammatory effects of cytokines. In addition, because it appears that reperfusion causes greater inflammation than ischemia per se, we investigated changes in cytokine antagonists in patients treated with thrombolytic agents compared with those in patients who did not receive them. Plasma neopterin was measured as a marker of macrophage activation.[17]

Table II. Clinical findings in the UA group

Patient no.	Age (yr)	Sex	Thrombolytic therapy
1	54	M	rTPA
2	62	M	UK
3	34	M	None
4	54	F	SK
5	58	M	None
6	64	F	None
7	51	M	None
8	63	M	rTPA

SK, Streptokinase; *UK*, urokinase.

METHODS

Patients. Thirty-four patients admitted to the coronary care unit (CCU) of the Ospedale Maggiore, Milan, for prolonged chest pain were included in this study. Twenty-six of these patients, 21 men and 5 women, 25-69 years old, had AMI confirmed by electrocardiogram (ECG) and an increase in the creatine kinase (CK) MB isoenzyme. In 8

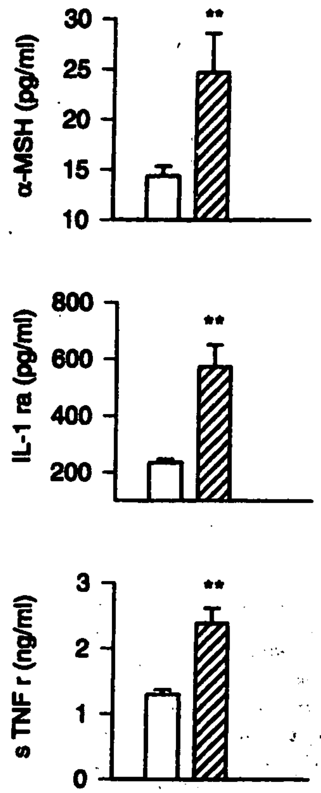

Fig. 1. Plasma concentrations of cytokine antagonists in patients with AMI or UA *(hatched bars)* on admission compared with those of control subjects *(white bars)*. Values are means ± SE. $^{**}p < 0.01$.

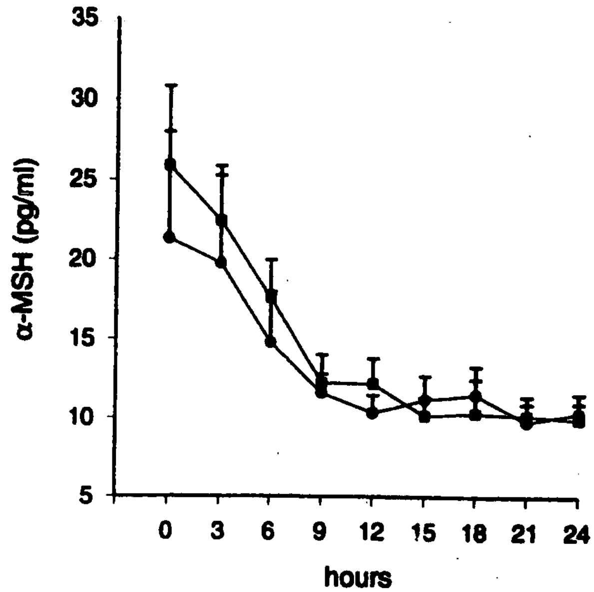

Fig. 2. Plasma α-MSH concentrations of samples taken at admission and every 3 hours thereafter for 24 hours were similar in patients with AMI *(squares)* and UA *(circles)*. Values are means ± SE.

patients, 6 men and 2 women, 51-84 years old, the final diagnosis on the basis of ECG findings and enzyme measurements was unstable angina pectoris (UA). Thrombolytic agents, either streptokinase (Streptase, 1.5 million U over a 1-hour period) or urokinase (Ukidan, 500,000 U over a 15-minute period followed by 250,000 U for 12-24 hours) or recombinant tissue-type plasminogen activator (rTPA) (Actilyse, 10 mg bolus followed by 50 mg during the first hour and 20 mg/hr during the next 2 hours) were administered in 26 patients. No patients were receiving long-term treatment with nonsteroidal antinflammatory drugs. Clinical characteristics of these patients are shown in Tables I and II. Two patients with AMI were excluded from thrombolytic treatment: one because of concurrent anticoagulant therapy and the other because of severe hypertensive crisis. Four other patients with prolonged chest pain did not receive thrombolytic therapy because their diagnosis on admission was UA. The time from the onset of chest pain, serum myocardial enzyme concentrations, acute-phase protein concentrations, white blood cell count, body temperature, ECG findings, and hemodynamic and electrophysiologic complications were recorded for each patient. Concomitant therapy included heparin, salicylic acid, and nitrates in all patients; β-adrenergic blocking agents were administered to 17 patients with AMI and 1 patient with UA. Calcium-channel blocking agents were given to 10 patients with AMI and 4 patients with UA. Control blood samples were obtained from 30 normal blood donors, 23 men and 7 women, 26-64 years old.

Protocol. Blood samples (10 ml in ethylenediamine tetraacetic acid) were obtained immediately after admission into the CCU, at 3-hour intervals for the first 24 hours, and subsequently every day for 4 days. Blood was immediately centrifuged, and aliquots of plasma were stored at –70° C. α-MSH in unextracted plasma was measured with a double-antibody radioimmunoassay (Milab, Malmö, Sweden); the lower limit of sensitivity of the assay is 5 pg/ml; the cross-reactivity with other propiomelanocortin peptides (corticotropin 1-24, corticotropin 1-39, β-melanocyte-stimulating hormone, and γ-melanocyte-stimulating hormone) is <0.002%. Plasma concentrations of IL-1ra and sTNFr (75 kDa) were determined with enzyme-linked immunosorbent assays (Quantikine, R&D Systems, Minneapolis, Minn., for IL-1ra and Bender MedSystems, Wien, Austria for sTNFr). The limits of detection for IL-1ra and sTNFr assays are 22 pg/ml and 0.08 ng/ml, respectively. Plasma IL-1β and TNF-α also were measured with enzyme-linked immunosorbent assays (Quantikine). The sensitivities of the assays are 4.5 pg/ml for IL-1 and 7.5 pg/ml for TNF. Plasma neopterin was measured with a double-antibody radioimmunoassay (Immutest Neopterin, Henning, Berlin, Germany). The normal value is 1.4 ± 0.6 ng/ml (mean ± SD).

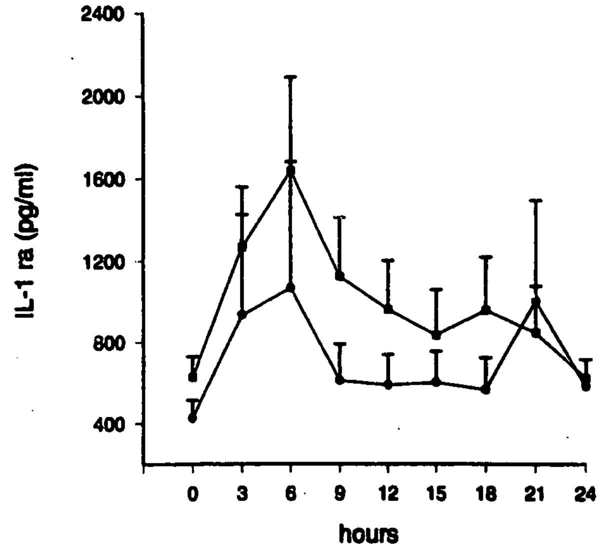

Fig. 3. Variations in plasma concentrations of IL-1ra were similar in patients with AMI *(squares)* and UA *(circles)*, but pattern of changes was different from that of circulating α-MSH. Values are means ± SE.

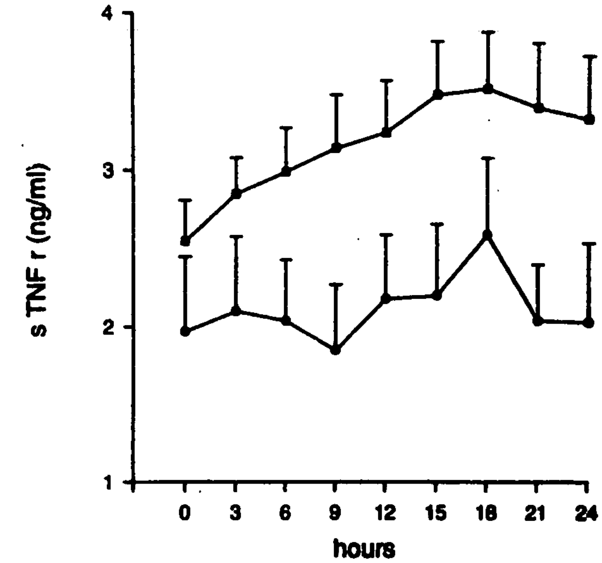

Fig. 4. Although plasma concentration of sTNFr tended to be greater in patients with AMI *(squares)* than in patients with UA *(circles)* there were no significant differences from UA values. Pattern of changes differed from that of plasma α-MSH (Fig. 2) and somewhat from that of IL-1ra (Fig. 3). Values are means ± SE.

Statistics. Results are expressed as means ± SE. Statistical analysis was performed by an omnibus analysis of variance for repeated measures followed by Tukey's *t* test for comparisons of individual means. Product-moment correlation coefficients were calculated to determine the significance of relations among certain measures.

RESULTS

Plasma cytokine antagonists in the AMI and UA groups. On admission to the CCU, patients with AMI had plasma concentrations of α-MSH, IL-1ra, and sTNFr that were significantly greater than those in control subjects (Fig. 1). Plasma concentrations of α-MSH were similar in the AMI and UA groups at presentation and decreased similarly in subsequent samples (Fig. 2). Initial concentrations of IL-1ra likewise were similar in the AMI and UA groups, but the pattern of changes in this cytokine antagonist over time differed from those in α-MSH. Mean plasma concentration of IL-1ra reached a peak 6 hours after admission and decreased thereafter (Fig. 3). Although patients with AMI tended to have IL-1ra values that were greater than those of subjects with UA, there was no significant difference between the two curves. sTNFr values also were slightly greater in patients with AMI than in patients with UA, but the differences were not statistically significant at any time (Fig. 4). The concentrations of all three cytokine antagonists were consistently low in samples taken on days 1-4. IL-1 was undetectable or at the lower limit of sensitivity of the method in all samples. Plasma TNF was detectable in approximately half of the patients, including two subjects with UA who had increased plasma concentration (13.5 and 30.8 pg/ml) of the cytokine.

Effect of thrombolytic treatment on plasma cytokine antagonists. There was no difference in the duration of chest pain in patients who received thrombolytic agents and those who did not (5.3 ± 0.8 hours and 3.8 ± 0.6 hours, respectively; $p > 0.05$). Plasma α-MSH concentrations in thrombolysis-treated and untreated groups differed significantly (Fig. 5); whereas concentrations of the peptide were increased in early samples from patients treated with a thrombolytic agent, they were consistently low in untreated patients. These observations suggested that an increase in α-MSH was associated with thrombolysis; however, prethrombolysis measurements were not available because thrombolytic therapy was performed in the emergency department before admission to the CCU. To determine whether thrombolysis was crucial to the marked increase in circulating α-MSH, we measured plasma α-MSH before and after thrombolysis in four additional patients with AMI (patients 23-26, Table I). Although α-MSH concentration was normal in these patients on ad-

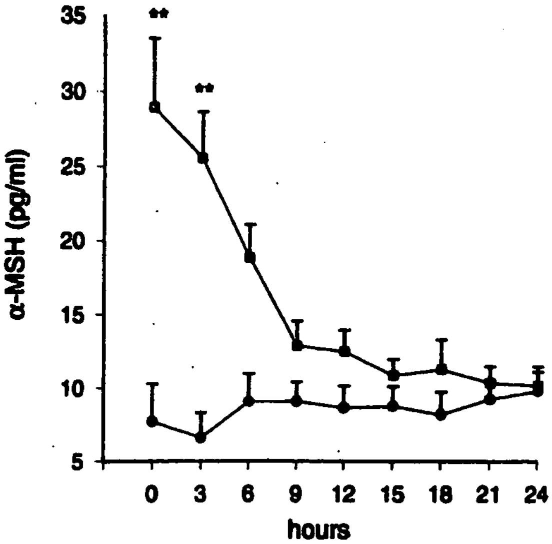

Fig. 5. In patients given thrombolytic agents (*squares*; $n = 24$), plasma α-MSH concentrations in samples taken on admission and 3 hours later were markedly greater than those of patients who did not receive thrombolytic agents (*circles*; $n = 6$). Values are means ± SE. $^{**}p < 0.01$.

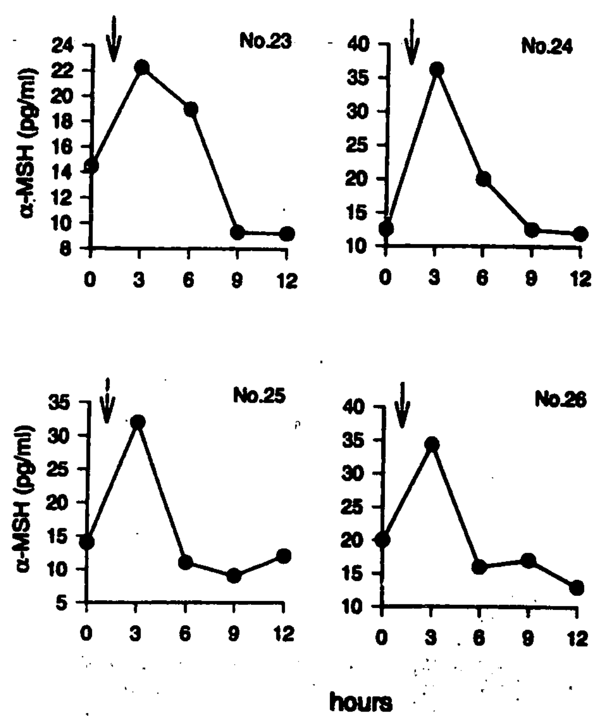

Fig. 6. Plasma α-MSH concentrations in samples taken on admission increased after treatment with rTPA (*arrows*) in four patients. Values are means ± SE.

mission, it markedly increased after thrombolytic treatment (rTPA), consistent with the idea that reperfusion rather than ischemia per se increases the plasma concentration of the hormone (Fig. 6). IL-1 ra concentrations likewise were greater 3 and 6 hours after treatment in patients who underwent thrombolysis than in those who did not (Fig. 7), whereas there was no significant difference in plasma sTNFr between the two groups (Fig. 8). Mean plasma neopterin, measured at 24 hours, also was greater in the thrombolysis-treated group (3.10 ± 0.7 ng/ml) than in the untreated group (0.46 ± 0.3 ng/ml) ($p < 0.01$). The proportion of patients with detectable plasma TNF was similar in the thrombolysis-treated and untreated groups (8 [33%] of 24 and 3 [50%] of 6, respectively).

Correlations between cytokine antagonists and enzymes released from injured myocardium. There were significant correlations between peak concentrations of cytokine antagonists and of enzymes released from injured myocardium in the AMI group although the peaks occurred at different times. The IL-1ra peak was positively correlated with the greatest concentration of CK ($r = 0.56$; $p = 0.007$), aspartate aminotransferase (AST) ($r = 0.76$; $p < 0.0001$) (Fig. 9), and lactate dehydrogenase (LDH) ($r = 0.65$; $p < 0.001$). Likewise there were significant correlations between sTNFr peak concentration and peaks in CK ($r = 0.48$; $p < 0.05$), AST ($r = 0.52$; $p < 0.05$), and LDH ($r = 0.47$; $p < 0.01$), whereas peaks in plasma α-MSH were correlated only with AST peaks ($r = 0.45$; $p < 0.01$).

DISCUSSION

These data show that α-MSH, IL-1ra, and sTNFr are released into the circulation during myocardial ischemia and thrombolytic treatment. It may be that these molecules reduce inflammation caused by cytokines and perhaps by other mediators of inflammation that are released by proinflammatory cells in the injured myocardium. If this explanation is correct, the mechanism is similar to protection against excessive cytokines at sites of inflammation in other inflammatory processes, such as in the synovial fluid of patients with rheumatoid arthritis, in which cytokines and their antagonists are found.[18]

Evidence from experimental models of acute myocardial ischemia indicate that inflammation associated with ischemia and reperfusion contributes to tissue damage.[19] Neutrophils are believed to be crucial determinants of inflammation after reperfusion of ischemic myocardium through their release of cytotoxic products.[20] Adhesion of neutrophils and monocytes to endothelial cells is an early event in the inflammatory response.[21] Cluster of differentiation (CD) leukocyte adhesion molecules CD11b/CD18

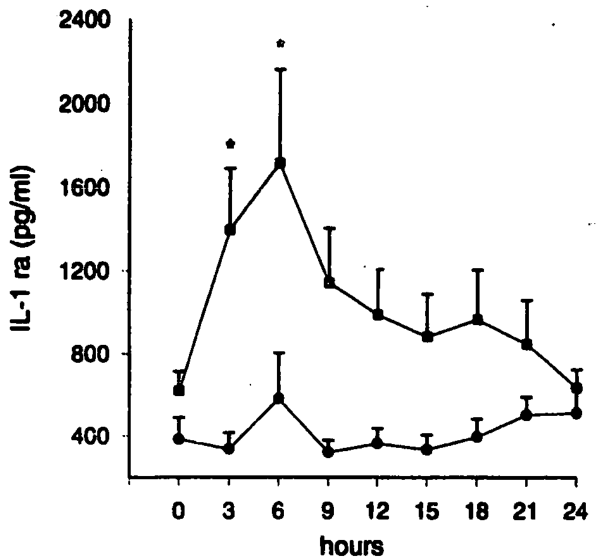

Fig. 7. Plasma IL-1ra was increased 3 and 6 hours after in patients given thrombolytic agents (*squares*; $n = 23$) and was greater than that of subjects not given thrombolytic agents (*circles*; $n = 6$). Values are means ± SE. $^{**}p < 0.05$.

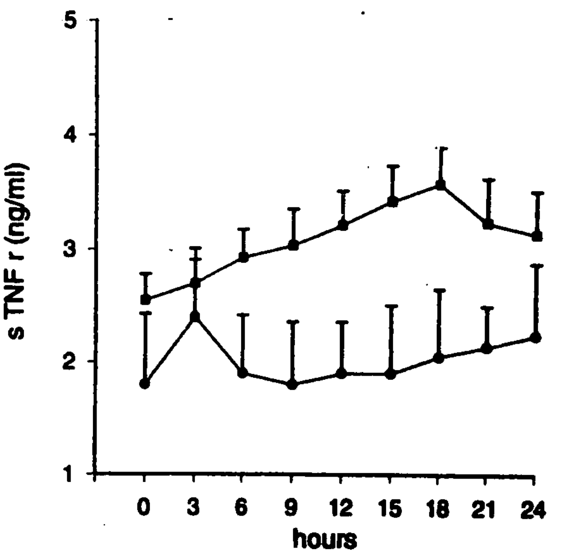

Fig. 8. Plasma sTNFr tended to increase progressively after thrombolytic agents were administered (*squares*; $n = 21$), but values were not significantly different from those in patients who did not receive these agents (*circles*; $n = 6$). Values are means ± SE.

(Mac-1) are up-regulated by chemotactic factors, including complement 5a (C5a) f-Met-Leu-Phe, IL-8, and PAF. Expression of Mac-1 starts within minutes f initiation of reperfusion. Locally produced cytokines, including TNF and IL-6, up-regulate CD11a/CD18 (lymphocyte function antigen–1 [LFA-1]) on leukocytes and intercellular adherence molecule–1 (ICAM-1) on endothelial cells. In contrast to the effects of chemotactic factors, this cytokine-induced increase in adhesion molecules is slow, peaking at 3 to 4 hours. Interactions of LFA-1 and Mac-1 with ICAM-1 are crucial to transendothelial migration; anti-CD18 monoclonal antibodies inhibit this migration. Patients with unstable angina show increased expression of Mac-1 in granulocytes and monocytes from coronary sinus.[22] That adhesion and migration of leukocytes promote myocardial damage is indicated by the effectiveness of anti-CD18 monoclonal antibodies in reducing infarct size in models of ischemia and reperfusion. Antibodies to ICAM-1 likewise reduce tissue infiltration and damage caused by neutrophils in reperfusion injury.[23]

Cytokine antagonists likely also inhibit the inflammation associated with myocardial injury. Concentrations of these antagonists in the plasma of patients with myocardial infarction have been found to be similar to those in patients with endotoxemia.[24] We suggest that these molecules represent a natural response to reduce inflammation during myocardial ischemia and reperfusion. In experiments in mice, α-MSH inhibited neutrophil chemotaxis into subcutaneous sponges injected with IL-1, TNF, and C5a.[25] Furthermore this potent antiinflammatory molecule significantly reduced neutrophil migration into the pulmonary tree in a mouse model of adult respiratory distress syndrome.[26] Preliminary in vitro observations suggest that α-MSH inhibits IL-8– and f-Met-Leu-Phe–induced chemotaxis of human neutrophils (Capsoni et al., unpublished data). Because chemotactic factors are crucial in the early induction of adhesion molecules on leukocytes, antagonism of these substances by the release of α-MSH may reduce reperfusion injury. This idea is supported by the prompt increase in the plasma concentration of the peptide after thrombolysis. However, α-MSH also antagonizes proinflammatory effects of cytokines, including those of IL-1β, TNFα, IL-6.[15] Therefore it may be that α-MSH inhibits later leukocyte adhesion and migration mediated by cytokine-induced LFA-1 and ICAM-1. Peaks in plasma IL-1ra occurred later in patients with acute myocardial ischemia. This finding is consistent with antagonism of IL-1–induced adhesion molecules.

An important question concerns the source of cytokine antagonists. Whereas it is clear that IL-1ra and sTNFr are produced at inflammatory sites, the source of α-MSH is not certain. Early research indicated that the intermediate lobe f the pituitary was the source of α-MSH in the circulation.[13] However, it

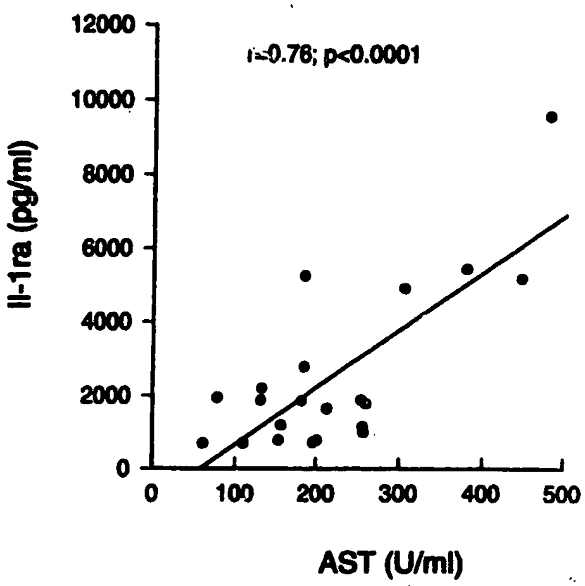

Fig. 9. Positive correlation between plasma IL-1ra and AST peaks in patients with AMI.

is now clear that the peptide also can be produced by several nonpituitary tissues.[13] We recently found that the concentration of α-MSH in the synovial fluid of patients with rheumatoid arthritis is greater than that in plasma.[18] This observation clearly indicates that this potent antiinflammatory peptide is produced within a site of inflammation. Furthermore the gene for the POMC precursor of α-MSH is expressed in the rat heart, and POMC is posttranslationally processed to α-MSH by cardiac muscle cells.[27] We suggest that inflammatory mediators released during reperfusion can release α-MSH from cardiac cells and thereby reduce chemotaxis and inflammation.

There was no difference in release of cytokine antagonists in patients with AMI or UA. This finding is consistent with recent observations by Liuzzo et al.[28] that patients with UA have increased plasma concentrations of C-reactive protein and amyloid A in the absence of myocardial necrosis. In their study, increases in these markers of inflammation at the time of hospital admission were associated with poor outcome. It appears that focal inflammation in the coronary arteries is involved in the genesis of unstable angina.[1] Therefore the inflammatory state of a coronary lesion may be a more important determinant of the clinical outcome than is the degree of stenosis.

We did not observe any increase in circulating IL-1, and plasma TNF increased in only a small number of patients. These findings are consistent with observations of the release ratio of cytokines and their antagonists: in general, production of IL-1ra exceeds that of IL-1.[29] It appears therefore that local production of cytokine antagonists is reflected in a definite increase in these molecules in the circulation, whereas cytokines exert their effects locally and their concentration in the general circulation increases only in systemic inflammation. Positive relations between plasma concentration of enzymes released by injured myocardium and anticytokine molecules in patients with AMI indicate that release of cytokine antagonists is proportional to infarct size.

Thrombolytic therapy and reinstitution of flow to the ischemic zone is an effective means of controlling ischemic tissue injury. However, because inflammation caused by ischemia and reperfusion is an important component of myocardial infarction, the treatment of patients with AMI also should include reduction of inflammation. Deleterious effects of high-dose corticosteroids in patients with AMI suggest that this antiinflammatory treatment should not interfere with the healing process.[30] We propose that α-MSH, which has potent anticytokine properties associated with a broad antiinflammatory spectrum, would be useful in this condition.

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Clinical and angiographic correlates of normal creatine kinase with increased MB isoenzymes in possible acute myocardial infarction

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A retrospective study of patients with possible acute myocardial infarction was conducted over a 2-year period to evaluate the clinical characteristics, angiographic findings, and in-hospital prognosis in patients with normal total creatine kinase (CK) activity and increased MB isoenzyme activity (CK-MB). Thirty-nine cases were identified (study group) and compared with cases of Q-wave ($n = 77$) and non–Q-wave ($n = 60$) infarctions. Compared with the Q-wave group, study group patients were older (67.5 ± 9.0 vs 60.8 ± 11.5 years; $p < 0.01$) and more often had previous diagnoses of coronary disease (52.6% vs 18.2%; $p < 0.01$) and peripheral vascular disease (28.9% vs 10.4%; $p = 0.02$). Angina (92.2% vs 65.8%; $p < 0.01$) and ST elevation (81.8% vs 13.2%; $p < 0.01$) were more common in the Q-wave group. Nearly identical clinical profiles and electrocardiographic findings were observed in the study and non–Q-wave groups. Angiographic analysis revealed a higher frequency of multivessel disease in the study group (89.6%) than in the Q-wave group (48.6%, $p < 0.01$) but no difference between the study group and the non–Q-wave group (79.6%; p not statistically significant). Left ventricular function and in-hospital complications were similar among groups. It is concluded that patients with normal total CK activity and increased CK-MB concentration represent a subgroup of patients with non–Q-wave infarction with a high prevalence of multivessel coronary disease. (Am Heart J 1995;130:211-7.)

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Serial measurement of concentrations of creatine kinase (CK) and its MB isoenzyme (CK-MB) remains

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REVIEW ARTICLE

ROLE OF TUMOR NECROSIS FACTOR IN THE PATHOGENESIS OF INTRAVASCULAR COAGULOPATHY OF SEPSIS: POTENTIAL NEW THERAPEUTIC IMPLICATIONS

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ABSTRACT. Tumor necrosis factor (TNF) induced by bacterial lipopolysaccharide (LPS) was shown to have an important role in precipitation of septic shock and disseminated intravascular clotting (DIC). At the endothelial level TNF down-regulates thrombomodulin (thus preventing protein C formation) and inhibits the production of tissue plasminogen activator (t-PA), thus impairing anticoagulant mechanisms. On the other hand, TNF up-regulates the production of procoagulant factors such as t-PA inhibitor (PAI), tissue factor and platelet activating factor (PAF). These effects create an imbalance between procoagulant and anticoagulant mechanisms, in favor of the former. TNF also activates polymorphonuclears (PMNs), and increases their chemotaxis and adherence to endothelial surfaces by up-regulation of specific endothelial (ELAM-1) and PMN (CDw18) adherence proteins. The damage inflicted by activated PMN to the endothelial cell promotes tissue factor exposure and PAI release, with initiation of the characteristic explosive coagulation process of DIC, facilitated by the dissociation between pro- and anticoagulant mechanisms induced by TNF. These newly discovered mechanisms precipitating septic shock and DIC enable consideration of new treatments for this condition as anti-TNF antibodies or TNF inhibitors, anti-ELAM-1 antibodies anti-tissue factor antibodies, administration of activated factor C, etc. These therapeutic approaches may revolutionize the treatment of septic shock and DIC in the next decade.
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Keywords: tumor necrosis factor; diffuse intravascular coagulation; septicemia

Hemorrhagic necrosis is a striking phenomenon that was first observed by Coley (1) almost a century ago in sarcoma patients with a concurrent infection. Fifty years after this pioneering observation, the factor responsible for initiating this coagulopathy was found to be endotoxin, the bacterial lipopolysaccharide (LPS) (2). However, it was not until O'Malley et al. (3) in 1962 and Carswell et al. (4) in 1975 observed that serum from LPS-injected animals, with (4) or without (3) priming with Bacillus Calmette Guerin, elicited hemorrhagic necrosis of transplantable tumors *in vivo*. The serum factor responsible for this phenomenon was given the name of tumor necrosis factor (TNF) (4).

Recent purification of TNF and determination of its sequence (5,6) as well as its subsequent availability as a pure recombinant material enabled scientists to establish, among the multiple biologic effects of TNF (reviewed in 7), its central role in sepsis and in precipitation of disseminated intravascular coagulation (DIC) and shock. The mortality associated with this type of fulminant sepsis remains above 50% (8,9) despite considerable improvement in antibiotic and supportive therapy. The poor outcome of these patients and the failure of corticosteroids to reduce this rate (10,11) called for a better understanding of the pathogenesis of sepsis and for therapeutic agents that might reduce the high mortality rate associated with

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this condition. The findings presented at the Second International Meeting on TNF and Related Cytokines in January 1989, and data published shortly thereafter, gave a better insight into the myriad, confusing and complex effects of TNF and have facilitated the identification and dissection of the mechanisms by which TNF mediates septic shock and DIC. As a result, new potential therapies are being considered.

In this review, I will summarize a) the evidence ascribing TNF a pivotal role in septic shock and DIC, b) the complex, highly coordinated events mediated by TNF, which lead to the development of a hypercoagulable state in sepsis resulting occasionally in DIC, and c) potential therapeutic interventions which may abort or reverse the highly lethal process of septic shock.

Normal Coagulation Pathways and their Regulation

Three closely linked biological components are involved in normal hemostasis: the endothelial cells of the blood vessels, platelets and coagulation proteins (Fig. 1).

The predominant pathway of coagulation in DIC (reviewed in 12) is the "extrinsic" one. This pathway is activated upon exposure of circulating factor VII to tissue factor. The tissue factor, present in endothelial cells (and monocytes) in a cryptic form, can be exposed upon cell damage or may be synthesized and expressed on the cell surface following activation of these cells with monokines such as TNF and interleukin-1 (IL-1) (13,14). Tissue factor expression is a vital event signaling the activation of the extrinsic clotting mechanism. It combines with factor VII and

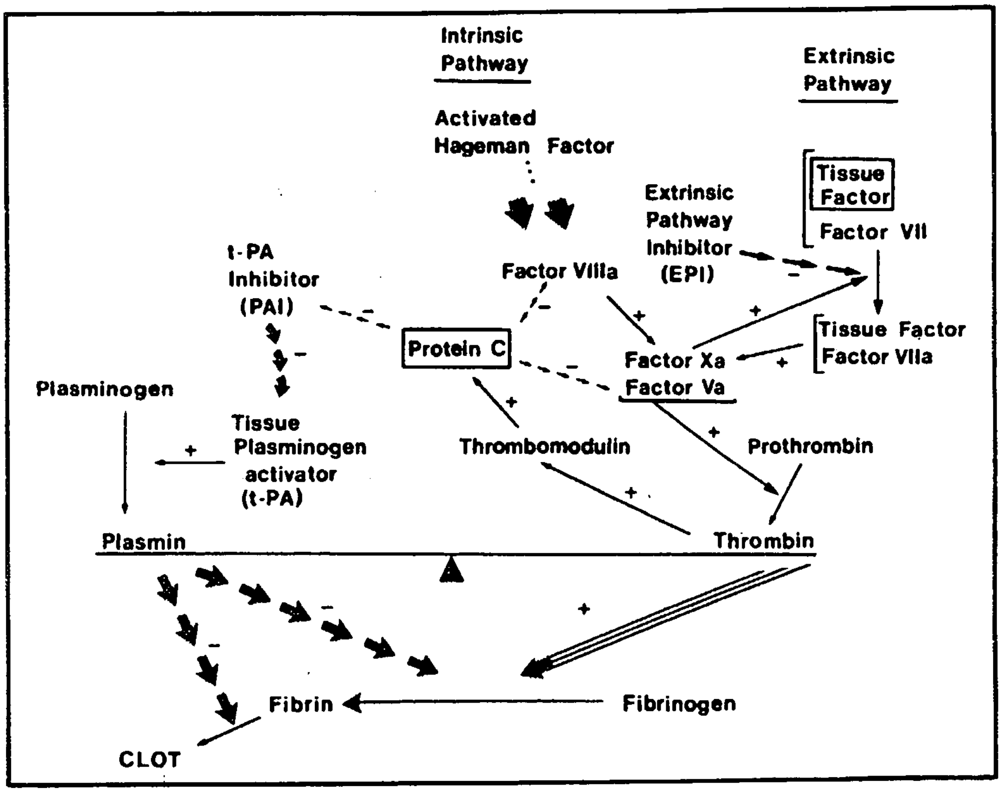

Fig. 1. Coagulation cascade induced by the extrinsic pathway activation and its inhibitors (relevant to this review). Release of tissue factor forms a complex with factor VII. This complex, after its activation, further activates factor X which aids a) thrombin formation and, b) together with the extrinsic pathway inhibitor (EPI), prevents further activation of factor VIIa/tissue factor complex. Thrombin, via thrombomodulin, induces protein C which inhibits activated factors VIII and V and, consequently, further thrombin formation. Protein C also inactivates tissue plasminogen activator inhibitor (PAI), thus enabling tissue plasminogen activator (t-PA) to convert plasminogen to plasmin, which will inhibit fibrin formation and will promote clot dissolution. (Broken arrows = inhibition; unbroken arrows = activation)

is converted into an active factor VII/tissue factor by minute amounts of pre-existing active factor X. A factor VIIa/tissue factor complex, thought to be the first enzyme/cofactor complex formed on the cell surface during blood coagulation *in vivo*, further activates factors X and IX. Factor Xa has two main functions: a) it serves as a positive feedback mechanism that further amplifies factor VII/tissue factor activation (15,16) (Fig. 1), and b) together with factor Va, phospholipids and Ca^{++} ions, it forms the prothrombin-converting principle responsible for thrombin generation. Thrombin converts fibrinogen to a clottable derivative, the *fibrin* monomer, which upon rapid polymerization forms a clot, stabilized by factor XIIIa.

These coagulation pathways are strictly controlled by highly efficient inhibitory mechanisms (Fig. 1). At the level of factor VII/tissue factor complex activation, a protease given the provisional name of extrinsic pathway inhibitor (EPI) combines with factor Xa, neutralizing its activity. This prevents factor Xa from further amplifying activation of the factor VII/tissue factor complex. Furthermore, the EPI/factor Xa complex combines with preactivated factor VIIa/tissue factor complex and inhibits it. This prevents promotion of coagulation via the extrinsic pathway (reviewed in ref. 16). A main counterbalancing mechanism to the procoagulant activity of thrombin (which forms fibrin from fibrinogen and thus promotes clot formation) is *plasmin* which degrades fibrin (by fibrinolysis) and inactivates is precursor fibrinogen. The formation of plasmin from plasminogen is facilitated by tissue *plasminogen activator* (t-PA) and urokinase. Endothelial cells secrete inhibitors for both urokinase and t-PA (see Fig. 1).

Another coagulation surveillance mechanism is induced by thrombin as a negative feedback loop (Fig. 1): thrombin complexes with thrombomodulin, a component of the luminal surface of the endothelial cell. This event activates *protein C*, which in turn inactivates the function of coagulation factors of the intrinsic (factor VIIIa) and the common coagulation pathways (factor Va). This prevents additional thrombin and clot formation. In addition, activated protein C degrades the *t-PA inhibitor* (PAI) (17), promoting plasmin formation and consequently activating fibrinolysis. Thus, protein C is a powerful anticoagulant, neutralizing active clotting factors while simultaneously intensifying clot degradation by augmentation of fibrinolysis (Fig. 1).

Role of TNF in Septic Shock

A considerable body of evidence has implicated TNF as one of the mediators of gram-negative (endotoxin) induced shock. Infusion of recombinant TNF into rats resulted in hypotension and metabolic acidosis, culminating in the death of the animal. At necropsy, ischemic and hemorrhagic lesions of the adrenals, pancreas, lungs and the gastrointestinal tract were observed, similar to those caused by endotoxin administration (18). All these changes were prevented if the animals were pretreated with polyclonal (19) or monoclonal (20) neutralizing antibodies to TNF, suggesting a role for TNF in the generation of these pathophysiological effects. Furthermore, mice treated with anti-TNF antibodies did not succumb to lethal endotoxin doses (19). The administration of neutralizing anti-TNF monoclonal antibodies to baboons *prior* but *not after* infusion of a lethal amount of *Escherichia coli* could also prevent shock and death (18). Interestingly, although the antibody-treated baboons were administered antibiotics only 12 h after the initiation of the experimental bacteremia, they exhibited no signs of toxicity compared with controls. This observation implies that the presence of bacteria in the bloodstream is not sufficient to cause toxicity related to sepsis and that host immune responses, such as TNF production, are required as well.

Evidence for a role of TNF in human septic shock was provided by studies of Waage et al. (21) and Girardin et al. (22). They showed a direct correlation between serum levels of TNF and the severity of meningococcemia. Thus, without exception, patients with a fatal outcome had serum levels of TNF above 130 pg/ml (23). Finally, it was shown in dogs that endotoxin or TNF alone can simulate the cardiovascular hemodynamic abnormalities of septic shock (24), although the mechanisms for this phenomenon remain to be elucidated.

Role of TNF in the Septic Coagulopathy

Recent studies suggest that TNF precipitates necrosis by disrupting the fine balance of the coagulation system, specifically, by dissociating the clotting mechanisms from their inhibitory surveillance. It has been documented that TNF activates the clotting mechanisms by up-regulating tissue factor formation (13,14). This can initiate the cascade of the extrinsic coagulation pathway (Fig. 1). In addition, human endothelial cells produce large amounts of platelet-activating factor (PAF) following their stimulation with TNF (25). Besides its role in activating platelets to aggregate and degranulate, PAF directly stimulates polymorphonuclears (PMNs) for chemotaxis, degranulation and superoxide radical production (reviewed in ref. 12) (Fig. 2). This facilitates endothelial damage by elastase (reviewed in ref. 26) predisposing to clot formation. PAF is thought

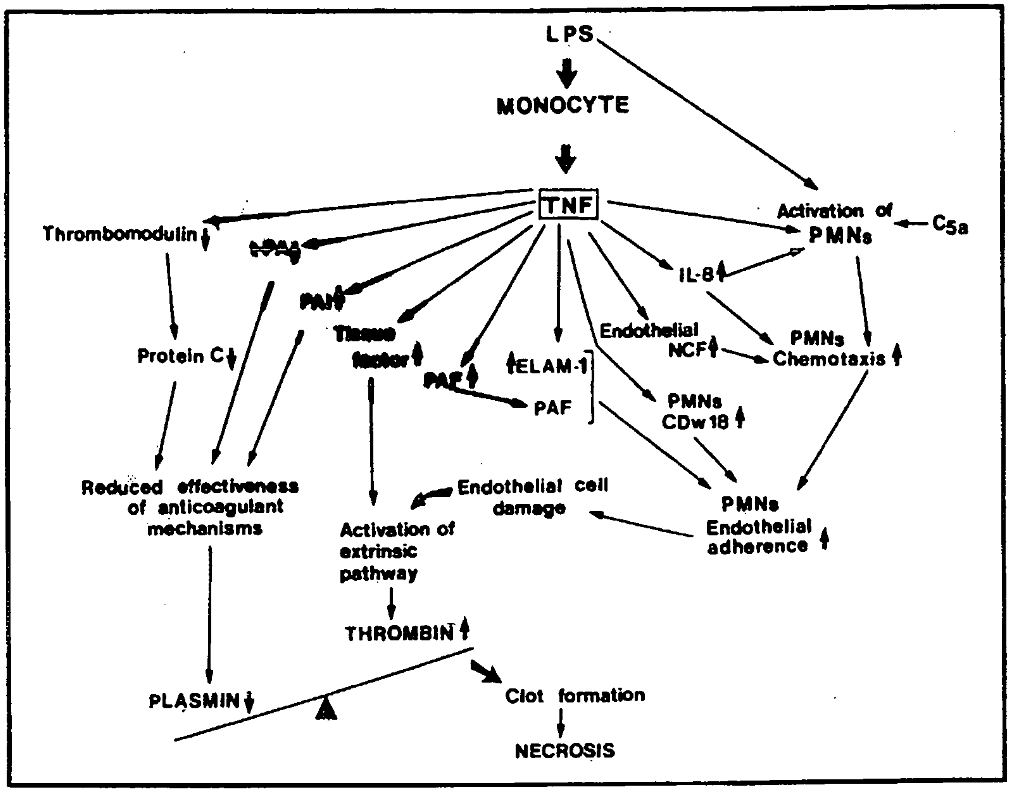

Fig. 2. Role of tumor necrosis factor in hemorrhagic necrosis. Lipopolysaccharide activates monocytes to release TNF. At the endothelial cell level, TNF down-regulates the thrombomodulin and the production of t-PA while up-regulating the production of PAI, tissue factor, platelet activating factor (PAF), endothelial neutrophil activating factor (NCF) and endothelial leukocyte adherence molecule 1 (ELAM-1). TNF also activates the polymorphonuclears (PMNs), and increases their chemotaxis through IL-8 and NCF and their surface adherence molecule -CDw18. The activated PMNs adhere to the endothelial cell and damage it, promoting tissue factor exposure and, thus, initiating coagulation through the extrinsic pathway. The concomitantly liberated PAI and the reduced protein C concentrations are responsible for the ineffectiveness of the anticoagulant mechanism. The imbalance between the low plasmin and high thrombin favors clot formation and necrosis. (Abbreviations as in Fig. 1.)

to have some role in experimental gram-negative sepsis as its infusion to animals elicited hypotension, decreased cardiac output and hypovolemic shock (27,28). All these effects were reversed by PAF receptor antagonists (28).

Simultaneous to the up-regulation of procoagulant activities, TNF down-regulates the anticoagulant mechanisms. Indeed, TNF was shown to inhibit thrombomodulin expression on the cell surface (13,14) by promoting thrombomodulin internalization and degradation (29). As a result, protein C activation, dependent on thrombomodulin, is severely impaired, and the coagulation mechanisms are devoid of a key anticoagulant. In the absence of protein C, activated clotting factors (Va and VIIIa) are not neutralized and the fibrinolytic mechanisms are adversely affected; as a result of protein C absence, PAI is not neutralized (Fig. 1). Furthermore, TNF directly *up-regulates* the PAI formation which, at this stage, remains intracellular (30,31), while concomitantly *down-regulating* t-PA generation. These events, which minimize plasmin formation, abolish the anticoagulant activity of the fibrinolytic mechanism, especially upon the subsequent release of PAI from the damaged endothelium. This leaves the scene open to an unopposed and uncontrolled procoagulant activity.

Role of Complement and Neutrophils in DIC

Even under the most favorable conditions for coagulation as described above, necrosis may not occur without the participation of PMNs and the complement fraction C5a. Indeed, elegant studies of Rothstein et al. (32,33) showed that hemorrhagic necrosis can develop only in an area intensely infiltrated with PMNs and that mice deficient in their ability to secrete the fifth component of the complement (C5) cannot develop hemorrhagic necrosis, despite adequate pretreatment with LPS and TNF, which induced local PMN accumulation.

Experiments in mice pretreated sequentially with LPS and 24 h later with TNF demonstrated the strictly necessary role of C5 in the initiation of the necrotic process (33): a) The ability of C5-deficient mice to express hemorrhagic skin necrosis in the above experiments was restored if they were administered plasma from C5-sufficient mice. b) Heat inactivation of the plasma of C5-sufficient mice abrogated the capacity of plasma to promote skin necrosis in C5-sufficient mice. c) Normal mice plasma depletion of C5b by zymosan (which does not affect C5a activity) did not impair the capacity of this plasma to supplement the C5a-deficient mice. However, the precise mechanism through which C5a facilitates necrosis remains an enigma.

As mentioned above, the neutrophils are a crucial component facilitating necrosis and, in their absence, DIC is aborted. Indeed, Thomas and Good (34) demonstrated almost 40 years ago that endotoxin cannot initiate DIC in animals rendered neutropenic by chemotherapy. However, such animals given leukocyte transfusion before LPS administration regained the capacity to manifest DIC (35). To participate in DIC, PMNs must first adhere to the endothelium. Endothelial leukocyte adhesion molecule-1 (ELAM-1), a recently identified and cloned cell surface glycoprotein receptor expressed by the endothelium (36), mediates the attachment of PMNs to the blood vessel lining; antibodies to this receptor domain were shown to effectively block PMN adherence. Following this adherence to the endothelial surface, activated PMNs produce and release oxygen radicals in the microenvironment adjacent to the neutrophil. These radicals oxidize and thus inactivate the potent leukocyte elastase inhibitors α_1-proteinase inhibitor and α_2-macroglobulin. These events create a sequestered physiologic milieu that enables elastase, the most powerful protease released by activated PMNs to damage the endothelium to which the PMNs adhere within the inflammatory focus (reviewed in 26). In addition, the oxygen radicals directly activate collagenase and gelatinase, two additional proteases released by PMNs. These proteases further assist elastase in its endothelial destructive activity (reviewed in 26).

This is the critical stage upon which the damaged endothelium expressing tissue factor (induced by TNF) initiates clotting via the extrinsic pathway and releases tissue PAI (also induced by TNF). PAI further intensifies this coagulation process by inhibiting fibrinolysis (see above and Fig. 2).

Recent evidence implies that recruitment, endothelial adherence and activation of PMNs are closely supervised and coordinated by TNF (Fig. 2): a) TNF (and IL-1) induces fibroblasts to release a newly purified cytokine also called neutrophil-activating factor (proposed name is IL-8), which promotes PMN chemotaxis and recruitment (37,38). b) TNF itself is chemotactic for PMNs (39) and also induces formation of a neutrophil chemotactic factor by endothelial cells (40). c) TNF supports the endothelial adherence of PMNs by induction of PAF (25) and by rapid up-regulation of endothelial surface proteins (such as ELAM-1) as well as neutrophils' surface proteins (such as CDw18), facilitating PMN adherence to endothelial cells (36,41–43), and d) before and after their endothelial adherence, PMNs are activated by TNF (44), PAF and C5a to produce oxygen radicals (reviewed in 12).

Thus TNF not only enhances the endothelial procoagulant potential but also promotes the adherence and activation of PMNs required for endothelial damage.

Overview of the Mechanism of TNF-induced Hemorrhagic Necrosis

Bacterial LPS activates a) monocytes to release TNF, and b) the complex cascade of complement. On one hand, TNF will induce endothelial cells to produce procoagulant factors (Fig. 2) such as tissue factor and PAF, while on the other hand it will down-regulate anticoagulant mechanisms by inhibiting thrombomodulin, thus preventing activation of protein C. Furthermore, TNF will inhibit fibrinolysis directly through down-regulation of t-PA and indirectly, by promoting *de novo* synthesis of tissue PAI (30,31).

Concomitantly, TNF will prepare the receptiveness of endothelium for neutrophils by inducing the expression of the neutrophil adhesion proteins ELAM-1 on the endothelial surface (36) and the CDw18 on the PMN membrane (43). These proteins can act as cell-associated signals for PMN adhesion (reviewed in ref. 12).

Simultaneous to the above-described influences of TNF on the endothelial cells, it will induce neutrophil activating factor (whose proposed name is IL-8)

(37,38), which will promote PMN chemotaxis and attraction to the endothelium already primed for PMN adhesion by TNF. This is the critical stage in the initiation of coagulation. Thus, the highly coordinated interplay between humoral components (C5a, coagulation/anticoagulation factors, chemotactic factors) and cellular components (monocytes, endothelium, neutrophils) culminates in neutrophil-mediated endothelial damage with plasma membrane exposure of tissue factor, responsible for the explosive coagulation process of DIC — all this under the strict regulation of TNF. Complicated as it is, the above mechanism is probably an oversimplification of the actual process as it occurs *in vivo*. It should be realized that TNF is a necessary, yet not a sufficient trigger of septic shock and DIC. In order to precipitate these conditions, TNF probably synergizes with other substances, such as LPS, IL-1, leukotrienes, prostaglandins, endorphins — just to name a few (for review see ref. 12). In the absence of these facilitating factors, TNF is relatively innocuous. One should not depict, therefore, the mode of action of TNF as an avalanche initiated by the mere exposure to TNF, and ending up in an unavoidable physiopathologic catastrophe. Instead, one should realize that TNF is supported by additional permissive or synergizing factors facilitating the precipitation of septic shock. More remains to be learned about these interactions to fully understand the pathogenesis of septic shock.

Can Septic Shock and DIC be Reversed or Prevented?

Will our better understanding of the pathologic processes triggered by TNF in septicemia (Fig. 2) lead to an improved therapeutic arsenal for septic shock and DIC, and a better outcome for these patients? To answer this question, the chain of events culminating in septic shock was simplified (Fig. 3) in order to show sites for potential new therapeutic interventions.

The first site for a possible pharmacologic intervention is elimination of LPS from the circulation (Fig. 3). Although the antibiotic polymyxin B was shown to bind avidly to LPS in aqueous solution, it binds LPS poorly in the presence of other proteins (45,46). Indeed, polymyxin B had no effect on levels of either bound or free plasma endotoxin (47). Despite this, administration of subtherapeutic doses of polymyxin B in experimental septicemia could moderate the course of the disease and improve survival (47,48). It is possible that the effects of polymyxin B were not the result of LPS elimination but were due to a reduction in PAI production (49) or to prevention of complement activation (50) as previously reported. Thus, although LPS elimination might be a desirable goal, since it potentiates TNF toxicity (32), no effective means are yet available, although the value of anti-LPS antibodies is now under study.

With regard to the second intervention site, inhibition of TNF production and release can be achieved by its potent transcription and translation inhibitors — the corticosteroids (reviewed in 7). These agents could, theoretically, prevent septic shock. Indeed, previous studies have shown beneficial effects of steroids in animal models of septicemia, provided their administration was not delayed beyond 30 min after establishment of experimental sepsis (51). At this early stage, TNF production could be inhibited *in vivo* as demonstrated in the recent studies by Remick et al. (52). However, a slight delay of only 20 min in the steroid administration will not reduce late TNF yields. This study provides an explanation why glucocorticoids are clinically inefficient in the treatment of septic shock, as shown also by previous carefully conducted clinical studies (10,11). Indeed, by the time patients develop clinical signs and symptoms of septic shock, they already have high

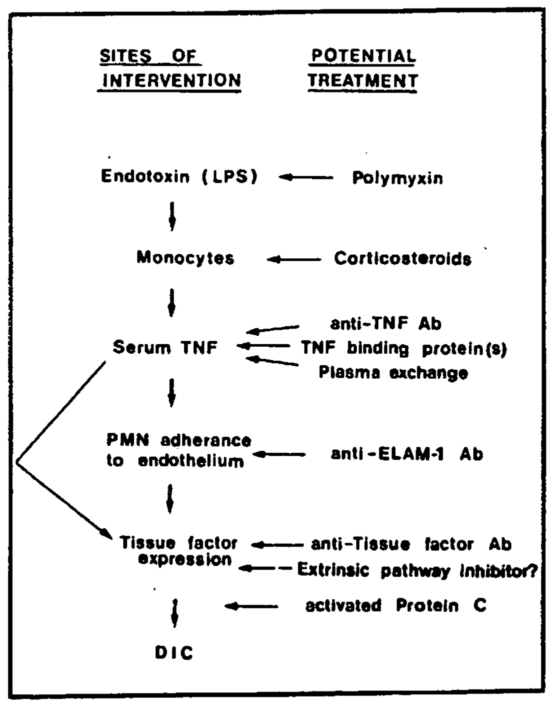

Fig. 3. Potential sites for medical intervention aimed to reverse or arrest the progression of septic shock and DIC (see also text). (Abbreviations as in Fig. 1.)

levels of circulating TNF (21,22). Furthermore, in a cell line, TNF was shown to reduce cytosolic steroid receptors (53). If this is a general phenomenon, then TNF may induce steroid resistance in septicemia, further reducing the ability of adrenocortical hormones to control TNF production.

A third potential site for a therapeutic intervention is at the level of circulating TNF (Fig. 3), specifically, neutralizing TNF with monoclonal antibodies. How-ever, it is both surprising and disturbing that anti-TNF antibodies proved effective in animal models only if administered *before* the injection of LPS or prior to the experimental establishment of bacteremia (18). The definitive value of such treatment will have to be determined by the administration of these neutralizing antibodies to septic shock patients and by evaluating their clinical response.

Neutralization of circulating TNF by a specific TNF inhibitor is another potential therapeutic intervention. Recently, such a natural TNF inhibitor was independently discovered by us in the urine of healthy persons (54), and by two other groups of investigators in the urine of febrile (55,56) and uremic patients (57).

The TNF inhibitor was purified to homogeneity. This enabled the determination of part of its biologic activities. This inhibitor binds TNF (57) and, remarkably, can even displace TNF from its specific receptors after binding (56). The inhibitor can prevent the cytotoxin activity of TNF (54), its ability to induce prostaglandin and collagenase in dermal fibroblasts and synovial cells (56), as well as the ability of TNF to induce cell-associated IL-1 (56) which can facilitate tissue destruction at the local level, thus precipitating clotting. Since this TNF inhibitor (given the provisional name of TNF binding protein — TBP) is expected to antagonize TNF actions at the level of the endothelial cells and neutrophils, it may abort TNF-mediated events precipitating DIC.

Is TBP a functional TNF inhibitor *in vivo*? A recent clinical observation in a renal failure patient with surprisingly minor symptoms, despite enormous blood concentrations of TNF, could be explained by the presence of putative TNF carrier proteins that might inactivate it (58,59). Since TBP was found to be the main TNF binding protein in the serum of renal failure patients (57), it is reasonable to assume that TBP could be the protective factor in the above case. However, the expectation that TBP will reverse septic shock should be guided by cautious optimism since TBP neutralizes TNF at a site similar to TNF antibodies (see Fig. 3) that were shown not to be effective in reversing experimental septic shock if administered after its precipitation (18). As with anti-TNF Ab, results of clinical studies with TBP in septic shock are eagerly expected.

Since circulating substances such as TNF are responsible for septic shock, the effect of plasma exchange vs. leukopheresis was examined in piglets (60). These studies showed that septicemia induced by *sublethal* doses of *E. coli* is followed by a rapid and persistent increase in TNF activity, and that plasma exchange, but not leukopheresis, lowered TNF levels, with improvement in the cardiac performance. When *lethal* doses of *E. coli* were administered and plasmapheresis was started 2 h later compared with plasma infusion only, plasma exchange significantly lowered serum TNF but had no effect on survival (61). Thus, the septic process advanced beyond the stage where simple TNF elimination was sufficient to arrest the course of the events. This important observation implies that attempts to inhibit a later, "downstream" event should also be evaluated.

Such an event is the adherence of PMNs to the endothelium, an event whose prevention by administration of antibodies to ELAM-1 may minimize the endothelial damage and abort the precipitation of the DIC. Further "downstream" is the tissue factor exposure on damaged endothelial cells (Fig. 3). Preliminary studies are encouraging since DIC could be prevented by administration of anti-tissue factor antibodies (62).

Can DIC be aborted by inhibition of the "extrinsic" pathway with its natural inhibitor (EPI) (Fig. 1)? Recent studies provided conflicting results regarding the EPI levels in sepsis (reviewed in 16). In addition, a lag in EPI/factor Xa-induced inhibition of factor VIIa/tissue factor *in vivo* (15,16) could explain why DIC would continue despite a normal plasma level of EPI in some septic patients.

Theoretically, EPI may emerge as a powerful tool in the treatment of DIC, provided that future studies will improve our ability to manipulate the EPI/factor Xa inhibitory kinetics for factor VIIa/tissue factor complex. Assuming that the tissue factor release could not be prevented, could replacement therapy with activated protein C restore the balance within the coagulation system and abort DIC?

Recent studies by Taylor et al. (68) have demonstrated that activated factor C can prevent DIC, tissue injury and death induced by an LD_{100} infusion of live *E. coli* in baboons. Future extension of these studies to human beings is expected to determine the effectiveness of this treatment in clinical practice.

CONCLUSION

Septic shock and DIC emerge as an over-response of the immune protective mechanisms to a bacterial insult. Moderation of this response by a spectrum of

new potential interventions used alone or in combinations may revolutionalize the therapeutic approach to septic shock and DIC in the next decade and hopefully will reduce the high mortality rate associated with this condition.

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ADDENDUM

Recent studies from our laboratory, confirmed by others, demonstrated that the TNF inhibitor (TBT) is actually the soluble TNF receptor (*EMBO Journal* 1990;9:3269–3273). Administration of this soluble TNF receptor to mice given lethal bacterial doses protected them from septic shock. DIC and death (Thompson et al., personal communication).

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Requests for reprints should be sent to L.B.J.

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PLASMA-FIBRINOGEN AND THROMBOEMBOLI AFTER MYOCARDIAL INFARCTION

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Summary In 120 patients with myocardial infarction subsequent non-fatal thromboemboli occurred only in patients in whom plasma-fibrinogen had exceeded 750 mg/dl. It is suggested that patients at risk from thromboembolism after infarction can be identified by monitoring plasma-fibrinogen and that appropriate anticoagulation might reduce morbidity.

Introduction

RAPID synthesis of fibrinogen by the liver is a non-specific response to myocardial infarction (M.I.),[1] surgery,[2] burns, fractures, and other injuries.[3] In all these conditions plasma-fibrinogen rises to a maximum between the 4th and 6th day after the event and returns to normal by the 10th to 14th day. Serious disorders may occur when the duration or degree of disturbance exceeds a biological limit. The result can be regarded as a breakdown of a defence mechanism, the original effects of which seem beneficial.[4]

We followed plasma-fibrinogen levels for a fortnight after M.I. and correlated the findings with non-fatal thromboembolic episodes.

Patients and Methods

120 patients with M.I. admitted consecutively to the coronary-care unit were studied. The diagnosis of M.I. required a typical history, serum-enzymes raised to twice the upper limit of normal, and acceptable electrocardiographic (E.C.G.) changes.

Patients were excluded if they were over 75 years of age or had established diabetes or renal or hepatic failure. We also excluded patients who had had surgical operations during the previous 2 months and those who died within 24 hours of admission.

Serum creatine kinase (C.P.K., normal up to 100 U/l), aspartate aminotransferase (G.O.T., normal up to 42 U/l), and lactate dehydrogenase (L.D.H., normal up to 525 U/l) were measured on the three mornings after admission. 10 patients had fewer than three measurements.

Plasma-fibrinogen was measured daily, whenever possible, for the first 6 days and thereafter every 2 or 3 days until discharge, by means of the Thorp-Stone nephelometer. Our normal range was similar to Cotton's—i.e., 250–350 mg/dl.[5] The highest (peak) measurement of enzymes and fibrinogen was the reference point to which we related subsequent events.

12-lead E.C.G.s were recorded on admission and recordings were repeated, with additional leads, when necessary. Other tests on admission included a chest X-ray, full blood-count, plasma urea and electrolytes, and serum lipids and uric acid. These were repeated and other investigations carried out when clinically indicated. All patients were seen by one of us regularly, but were clinically managed by the admitting physician. Whenever possible patients were out of bed by the 5th day and discharged by the 14th day. Anticoagulants were not routinely prescribed. 2 patients received anticoagulants because of their history, and those who sustained thromboemboli had anticoagulants after the event in the absence of contraindications.

Events

Death within 14 days of infarction.

Non-fatal cardiac arrests.—Patients with ventricular fibrillation or asystole who were successfully resuscitated.

Non-fatal thromboembolic episodes.—Our criteria for thromboemboli were strict and all were clinically obvious and potentially disabling. They comprise: (a) pulmonary infarction requiring two of the following three conditions, typical history of sudden pleuritic pain, with pleural rub or hæmoptysis, X-ray changes compatible with infarction, and a rise in L.D.H. or serum-bilirubin; (b) cerebrovascular accident (C.V.A.), which was only diagnosed when there was a major deficit such as hemiplegia or aphasia; (c) peripheral arterial occlusion with a loss of peripheral pulse with pain, paræsthesia, and paralysis.

Results

Of the 120 patients 97 were men and 23 were women and the average age was 56·7 years.

Events

10 patients had a major non-fatal thromboembolism.

TABLE I—NUMBER AND TYPE OF THROMBOEMBOLIC EPISODES AND TIME OF OCCURRENCE AFTER M.I.

Type of episode	No. of patients	No. of days after M.I.	Mean age (yr)
C.V.A.	5	4, 4, 6, 6, 13	57·6
Pulmonary infarct	4	3, 3, 3, 10	56·5
Peripheral embolus	1	12	57

TABLE II—MEAN PEAK ENZYME CONCENTRATIONS IN PATIENTS IN WHOM THERE WERE R WERE NOT EVENTS AND STATISTICAL SIGNIFICANCE OF THE DIFFERENCE BETWEEN EACH SUBGROUP AND THOSE IN WH M EVENTS DID NOT OCCUR

Type of event	No. of patients	Mean peak enzyme concentrations					
		C.P.K.		G.O.T.		L.D.H.	
		Mean ± S.E.	P	Mean ± S.E.	P	Mean ± S.E.	P
Deaths	10	1758 ± 178	<0·02	484 ± 94	<0·05	3801 ± 530	<0·01
Non-fatal cardiac arrests	6	2030 ± 290	<0·05	401 ± 75	<0·1	2706 ± 341	N.S.
Non-fatal thromboemboli	10	1832 ± 176	<0·01	378 ± 34	<0·01	2905 ± 168	<0·001
No events	94	1205 ± 63	..	237 ± 13	..	1841 ± 71	..

Table I gives details of the type and timing of the episodes. There were 10 deaths and 6 non-fatal cardiac arrests.

Enzyme Concentrations and Events

Table II shows the peak enzyme concentrations in patients in whom events did or did not occur. Although all three enzyme concentrations were significantly increased in patients experiencing events, we confined further discussion to C.P.K. concentrations because both G.O.T. and L.D.H. can be affected by secondary changes arising in liver and lungs. Table III shows the number of events in three groups with increasingly high peak C.P.K. levels.

TABLE III—DISTRIBUTION OF EVENTS IN GROUPS OF PATIENTS WITH INCREASING PEAK C.P.K. CONCENTRATIONS

—	Range of peak C.P.K. values (U/l):		
	up to 999	1000–1599	1600+
No. of patients	39	39	42
No. of deaths	1	3	6
No. of arrests	0	2	4
No. of thromboemboli	1	3	6
Total events	2	8	16

Plasma-fibrinogen and Events

In all but one patient (mentioned later) plasma-fibrinogen rose after M.I. In those who survived, plasma-fibrinogen reached a peak around the 5th post-infarction day, returning towards normal levels by about the 10th day.

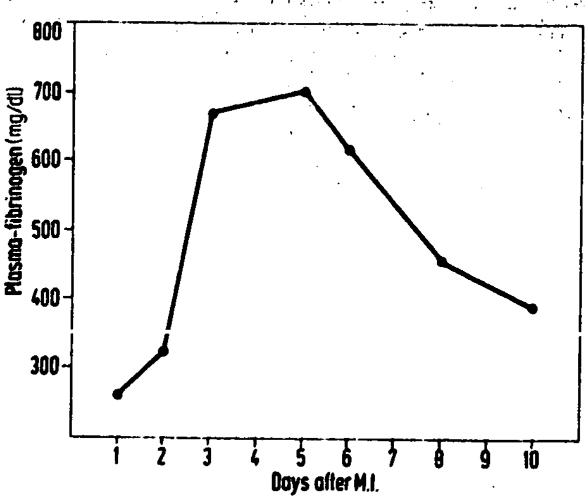

Fig. 1—Daily plasma-fibrinogen concentrations in a typical patient.

Fig. 1 shows plasma-fibrinogen concentrations in a typical patient. Fig. 2 shows the average daily concentrations in patients in whom no events occurred and in those with thromboemboli. The curves are flatter than in most individuals because of variation in the time at which peak values were reached.

TABLE IV—MEAN PEAK PLASMA-FIBRINOGEN IN PATIENTS IN WHOM EVENTS DID OR DID NOT OCCUR AND STATISTICAL SIGNIFICANCE OF DIFFERENCE BETWEEN EACH SUBGROUP AND THOSE IN WHOM EVENTS DID NOT OCCUR

Type of event	No. of patients	Mean peak fibrinogen ± S.E. (mg/dl)	Significance
Deaths	10	745 ± 75	N.S.
Arrests	6	703 ± 58	N.S.
Thromboemboli	10	937 ± 36	P<0·001
No events	94	691 ± 17	..

The average peak value for all patients was 717 mg/dl and was not significantly different in patients who died or had non-fatal cardiac arrests. The average peak value for patients with thromboemboli was 937 mg/dl, and this value is significantly higher than that in all other patients (P<0·0001).

Table V shows the number of events in three groups with increasingly high peak plasma-fibrinogen concentrations. Nine out of ten thromboemboli occurred in the

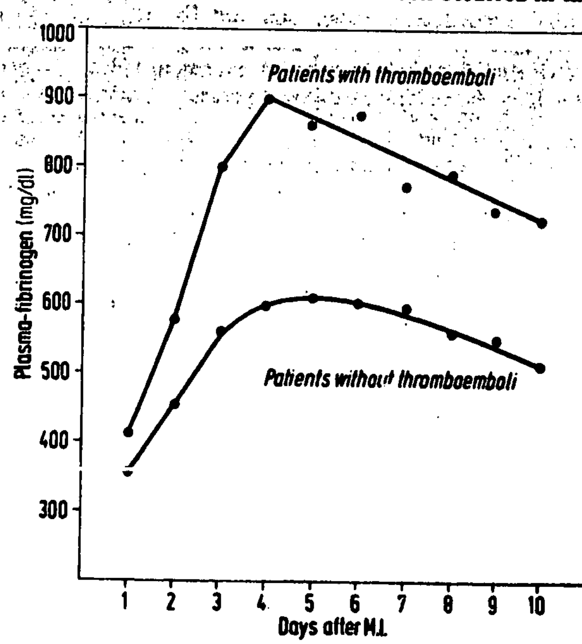

Fig. 2—Mean daily plasma-fibrinogen in patients experiencing and not experiencing thromboembolic episodes.

TABLE V—DISTRIBUTION F EVENTS IN GR UPS F PATIENTS WITH INCREASING PEAK PLASMA-FIBRINOGEN CONCENTRATIONS

—	Range of peak fibrinogen (mg/dl)		
	Up to 649	650–799	800+
No. of patients	40	40	40
No. of deaths	2	4	4
No. of cardiac arrests	2	3	1
No. of thromboemboli	0	1	9
Total events	4	8	14

group with the highest concentrations (over 800 mgm/dl)—the tenth occurred in a patient with a peak value of 750 mg/dl. Fibrinogen concentrations did not correlate with other events.

Timing of Events in Relation to Peak Plasma-fibrinogen

Deaths.—3 patients died before the 5th day. 1 of these, a woman aged 34, had had an M.I. 18 months before. She had cardiogenic shock and evidence of vascular occlusion of the left leg. Concentrations of plasma-fibrin-degradation-products were high (>40 μg/ml) in this patient and plasma-fibrinogen was low (324 mg/dl) on the one occasion it was measured. There were other coagulation abnormalities which suggested that disseminated intravascular coagulation could account for the low plasma-fibrinogen concentrations in this patient.

Non-fatal cardiac arrests all occurred before the 5th day.

Thromboemboli.—In 4 patients, thromboemboli occurred before plasma-fibrinogen had reached its peak value. However, the last plasma-fibrinogen measurement before the event in these patients was 1020, 925, 850, and 650 mg/dl. Thus only one thromboembolic event occurred when the plasma-fibrinogen concentration had not already reached 750 mg/dl. In this patient, the fibrinogen rose from 650 on the 3rd day to reach a peak of 970 mg/dl on the 6th day.

In patients in whom thromboemboli developed early plasma-fibrinogen continued to rise until the 5th or 6th day, but there was no suggestion of a significant secondary rise after the event.

Correlation between Peak Enzyme Values and Peak Fibrinogen Concentrations

There was a positive correlation between peak enzyme and peak fibrinogen concentrations. The coefficients of correlation were 0·44, 0·29, and 0·40 between fibrinogen and C.P.K., G.O.T., and L.D.H., respectively. All these coefficients are statistically significant (P<0·002).

Discussion

Thromboemboli have been reported after myocardial infarction in up to 30% of patients.[6,7] Early mobilisation has undoubtedly reduced the frequency of venous thrombosis and none of our patients with pulmonary infarction had clinical evidence of deep-vein thrombosis. But early mobilisation cannot be expected to influence intracardiac clotting and subsequent formation of emboli, and 3 of our patients with C.V.A.s were ambulant at the time of the incident. Anticoagulants, once routine treatment, have tended t become less popular, but there are still patients who may need them. The problem is to identify the group at risk.

Clinically recognisable features which might encourage thrombosis and formation of emboli after M.I. include a drop in blood-pressure, arrhythmias, hæmoconcentration, and poor cardiac output, but none of these factors was more common in our patients with thromboemboli than in the other patients. 1 patient had supraventricular tachycardia and a short episode of atrial fibrillation 6 hours after pulmonary infarction. Another had transient 2/1 heart block 12 days before a C.V.A. A 3rd had a brief episode of ventricular tachycardia on the day before a pulmonary infarct.

Serum-enzyme concentrations have been said to reflect the size of myocardial infarction[8,9] and have been suggested as a useful prognostic indicator of survival.[10] Our findings suggest that thromboemboli are usually associated with high serum-enzymes and presumably large infarcts (tables II and III). One could regard a high serum-enzyme concentration as an indicator of patients at risk from thromboemboli, but although there is a positive correlation between enzymes and fibrinogen, there is considerable individual variation. We consider that a high plasma-fibrinogen is a more specific indicator of possible thromboembolism (table IV).

A stepwise regression procedure indicated that in order to discriminate satisfactorily between patients with thromboemboli and the rest, only fibrinogen was required in the discriminant function (P<0·005). Serum-enzymes did not improve discrimination. Furthermore, C.P.K. (and G.O.T.) rises and falls much more rapidly than fibrinogen and the peak is therefore more likely to be missed.

All 10 thromboemboli occurred in the 51 patients with a peak plasma-fibrinogen value of 750 mg/dl or more—a frequency of 20%. There were no thromboemboli in the other 59 survivors in whom plasma-fibrinogen did not reach 750 mg/dl.

Plasma-fibrinogen correlates positively with increased plasma viscosity,[11,12] which in itself could encourage clotting. Together with the increase in fibrinogen after M.I. there is also an increase in thromboplastin generation,[13] an increased rate of platelet utilisation,[14] and an increase in thrombin/thromboplastin activity.[15] All these changes indicate a general coagulation disturbance. The increase in plasma-fibrinogen may simply be an indicator of these diverse changes. Sevitt[4] considered that post-traumatic effects on coagulation could be divided into physiological responses which do not require intervention and exaggerated responses with pathological sequelæ which do. We suggest that a plasma-fibrinogen concentration of 750 mg/dl can be regarded as the dividing-line. Monitoring plasma-fibrinogen by nephelometer is simple compared with scanning of limbs and lungs by means of labelled fibrinogen and in addition it appears to identify patients in whom the development of intracardiac clotting and thromboemboli is more likely.

We have, of course, no proof that the C.V.A.s we encountered were all embolic, but the average age and history were no different from those in other patients. Alth ugh the incidence of C.V.A. is low (4%), the results are often catastrophic and well worth avoiding if possible.

As a result of our study, we have modified our attitude to anticoagulants. Unless there is a definite contraindication, we now give every patient with myocar-

dial infarcti n subcutaneous heparin (10 000 units twice daily) for 6 days, and a loading dose of warfarin on the 4th post-infarcti n day.

Neither heparin nor warfarin affect plasma-fibrinogen concentrations. If plasma-fibrinogen starts to fall without reaching 750 mg/dl we stop anticoagulation. If it reaches 750 mg/dl we maintain anticoagulation with warfarin for 6 weeks. The decision to stop or maintain anticoagulation can nearly always be made by the 6th day after infarction and often considerably earlier in patients in whom plasma-fibrinogen rises steeply. We chose to use heparin because it avoids the necessity of laboratory control and the subcutaneous route because it allows the patient freedom of movement. Our results so far have been most encouraging. We suggest that monitoring plasma-fibrinogen offers a rational basis for the use of anticoagulants after myocardial infarction.

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TREATMENT OF NEUROGENIC ORTHOSTATIC HYPOTENSION WITH A MONOAMINE OXIDASE INHIBITOR AND TYRAMINE

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Summary Six patients with neurogenic orthostatic hypotension were treated with a chemical preparation of tyramine and tranylcypromine ('Parnate'), a monoamine oxidase inhibitor (M.A.O.I.). Four had autonomic failure with no other neurological deficit (idiopathic orthostatic hypotension), and in two patients other neuronal systems were also involved (Shy-Drager syndrome). Previous therapy with fludrocortisone, ephedrine, elastic garments, postural training, and, in one patient, an anti-G suit was unsatisfactory. Tyramine given orally with tranylcypromine produced a moderate rise in blood-pressure which was sustained for 2–4 hours, enabling patients to walk about without symptoms of orthostatic hypotension. Measurement of circulating adrenaline and noradrenaline during therapy suggested that release of noradrenaline caused the pressor response. In three patients there has been a pronounced improvement for 8, 20, and 30 months. In a further patient, therapy has been successful in treating the orthostatic hypotension, although his mobility has been restricted by cerebellar ataxia. In one patient a confusional state developed during treatment and therapy was stopped. The only patient in whom the drugs did not produce a pressor response had orthostatic hypotension with failure of noradrenaline release. It is suggested that the pressor response to a M.A.O.I. and tyramine should be examined in patients with neurogenic orthostatic hypotension and that this treatment should be tried in those who respond.

Introduction

Disabling neurogenic orthostatic hypotension may occur in many diseases, and may also be a result of degenerative disease in the nervous system which is not related to any well-known primary cause.[1 2] It is due t dysfunction in autonomic pathways and was originally regarded as a medical curiosity but is now more frequently recognised either alone (idiopathic orthostatic hypotension) or together with degeneration of other neuronal systems (multiple system atrophy; Shy-Drager syndrome).[2] Many methods of treatment of neurogenic orthostatic hypotension have been described including the use of vasoconstrictor drugs, blood-volume expanders, elastic garments, and postural training.[3–6] In a few patients such therapy meets with success but this is often partial or short-lived.[6] Treatment with tyramine-containing food given with a monoamine oxidase inhibitor (M.A.O.I.), did not give consistent results, perhaps because the dose of tyramine in food is variable.[7–9] We used a chemical preparation of tyramine in capsules in combination with an M.A.O.I. ('Parnate' tranylcypromine,) to treat six patients with neurogenic orthostatic hypotension.

Patients and Methods

Six patients (two female, four male, aged 25–72 years) with pronounced orthostatic hypotension were treated. Patients had had symptoms of dizziness and syncope for from 3 to 21 years. On examination four of the patients had no evidence of other neurological disease and two patients had other neurological deficits—parkinsonism (case 5), cerebellar ataxia and evidence of corticospinal dysfunction (case 6).

The following investigations were carried out and were normal in all patients—erythrocyte-sedimentation rate; hæmoglobin, differential cell-count; blood urea and electrolytes (Na^+, K^+, Cl^-, Ca^{++}, PO_4^-); serum alkaline phosphatase and bilirubin; flocculation tests; B_{12}; Wassermann reaction; glucose tolerance test; urinary excretion of 17-ketosteroids, 17-hydroxycorticosteroids, and porphyrins; and 24-hour urinary excretion of metadrenalines.

Tests of autonomic reflexes controlling blood-pressure, sweating, and heart-rate were performed. The tests have been reviewed by Johnson and Spalding.[1] The abnormality f the baroreceptor reflex arc controlling blood-pressure was due t afferent block in ne patient and to afferent and efferent failure in another. In four patients the lesion was efferent and in one of these it was due to failure of noradrenaline release (table I).